Physiology of *Listeria monocytogenes* in relation to food components and biopreservation

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Physiology of *Listeria monocytogenes* in relation to food components and biopreservation

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Stellingen

1. Osmoregulatie in *Listeria monocytogenes* kan allerminst met een korreltje zout genomen worden.
   - Dit proefschrift, hoofdstukken 4, 5 & 6.

2. Het feit dat carnitine betrokken is bij het vetzuurmetabolisme van eukaryoten betekent niet automatisch dat het nuttigen van levensmiddelen die rijk zijn aan carnitine je slank maakt.
   - Flair "Pas ontdekt: carnitine maakt je slank", maart 1995.
   - Bieber, L.L. "Carnitine", Annu. Rev. Biochem. 57:261-283, 1988.

3. Het vermogen om de moleculaire samenstelling van de membraan aan te passen teneinde te overleven bij lage omgevingstemperaturen is niet voorbehouden aan bacteriën.
   - Russell, N.J. "Mechanisms of thermal adaptation in bacteria: blueprints for survival", Trends Biochem. Sci. 9:108-112, 1984.
   - NRC Handelsblad "Karpers verlengen en oxyderen vetzuren als het koud wordt", 15 februari 1996.

4. Onderstaande onderzoekers gaan voorbij aan de mogelijkheid dat nisineresistentie in *Listeria* spp. gebaseerd kan zijn op een verandering in de kopgroepsamenstelling van de cytoplasmatische membraan.
   - Mazzotta, A.S., and T.J. Montville. "Nisin induces changes in membrane fatty acid composition of *Listeria monocytogenes* nisin-resistant strains at 10°C and 30°C", J. Appl. Microbiol. 82:32-38, 1997.
   - Maisnier-Patin, S., and J. Richard. "Cell wall changes in nisin-resistant variants of *Listeria innocua* grown in the presence of high nisin concentrations", FEMS Microbiol. Lett. 140:29-35, 1996.

5. De conclusie van Abdel-Bar en Harris dat gekoelde salades kunnen worden geconserveerd door fermentatie met een vertegenwoordiger uit de groep van thermobacteria kan niet juist zijn.
   - Abdel-Bar, N.M., and N.D. Harris. "Inhibitory effect of *Lactobacillus bulgaricus* on psychrotrophic bacteria in associative cultures and in refrigerated foods", J. Food Protect. 47:61-64, 1984.
   - Pot, B., W. Ludwig, K. Kersters, and K.-H. Schleifer. "Taxonomy of lactic acid bacteria", p. 13-90. In L. De Vuyst, and E.J. Vandamme (ed.), "Bacteriocins of lactic acid bacteria", Chapman & Hall, London, 1994.

6. Het betoog van Borst dat fikse aantallen *Salmonella* bacteriën in melkpoeder nodig zijn om via flesvoeding gastro-enteritis bij baby's te veroorzaken staat op losse schroeven wanneer de resultaten van Lehmacher *et al.* in ogenschouw worden genomen. Deze onderzoekers vonden dat extreem lage aantallen van salmonellae (0,04 to 0,45 organismen per gram product) voldoende zijn om een grote *Salmonella* epidemie te veroorzaken, waarbij vooral kleine kinderen het slachtoffer waren.
   - Borst, P. NRC Handelsblad "Opwinding rond *Salmonella*", 8 februari 1997.
   - Lehmacher, A., J.Bockemuller, and S. Aleksic. "Nationwide outbreak of human salmonellosis in Germany due to contaminated paprika and paprika-powdered potato chips", Epidemiol. Infect. 115:501-511, 1995.

7. De maatschappelijke acceptatie van levensmiddelen die geproduceerd worden via moderne biotechnologie kan gestimuleerd worden door deze producten goedkoper aan te bieden dan de vergelijkbare traditionele levensmiddelen.

8. Verhoging van de BTW op het autogebruik teneinde het milieu te ontzien is een verkapte manier van de staat om geld binnen te halen aangezien iedere politicus weet dat Nederlanders hierdoor hun auto toch niet laten staan.

9. Onderzoek aan *Listeria monocytogenes* is interessant en relevant.
   - Dit proefschrift.
   - De Volkskrant, "België neemt Franse kazen uit handel", 20 september 1997.

Stellingen behorende bij het proefschrift van Annette Verheul:
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Contents

Abstract

Listeria monocytogenes is an important foodborne pathogen that has been responsible for severe infections in humans. The ubiquitous distribution of *L. monocytogenes* in the environment and its ability to grow at refrigeration temperature and at high osmolarity are of paramount importance for its hazardous status in minimally processed convenience foods. This thesis deals with the physiology of *L. monocytogenes* with focus on peptide utilization, osmoregulation and bacteriocin-resistance.

The growth of the protease-negative and multiple amino acid auxothropic *L. monocytogenes* was shown to be stimulated in the presence of proteolytic microorganisms or enzymes in a medium with casein as nitrogen source. It was subsequently demonstrated that *L. monocytogenes* is able to accumulate peptides consisting of up to eight amino acid residues via two different transport systems, a proton motive force driven di-tripeptide carrier and an ATP-dependent oligopeptide transporter. The accumulation of the peptides prolyl-hydroxyproline, prolyl-glycine and prolyl-prolyl-glycine, which are prevalent in collagen, contributed to osmotolerance in *L. monocytogenes* suggesting a role for the di-tripeptide tranporter in osmoregulation.

Salt tolerance in *L. monocytogenes* can also be invoked by betaine and L-carnitine, which are present in high concentrations in foods of plant and animal origin, respectively. Transport studies revealed that betaine and L-carnitine are acccumulated via separate highly specific uptake systems. Both transporters have high scavenging capacity for their substrates and are driven by the electrochemical gradient and ATP, respectively. It was evidenced that internal betaine inhibited both betaine and L-carnitine uptake and, similarly, internal L-carnitine inhibited both betaine and L-carnitine transport. The *trans* inhibition of both transporters was relieved upon osmotic upshock, which suggests that changes in membrane tension affect the binding pockets of the transporters. Furthermore, it was manifested that the betaine and L-carnitine transport systems are important in cold adaptation of the psychrotrophic *L. monocytogenes*.

The addition of bacteriocins produced by lactic acid bacteria, such as nisin and pediocin, might reduce *L. monocytogenes* numbers in foods, but may in addition select for bacteriocin-resistant variants. It was demonstrated that nisin resistance in a variant of *L. monocytogenes* Scott A, which gained its resistance by increasing stepwise exposure to nisin, was attributable to a reduction in the diphosphatidylglycerol content of the cytoplasmic membrane. Subsequent studies with five *L. monocytogenes* strains which all acquired nisin resistance by training, revealed that these mutants exhibited phenotypic characters that differed from that of the parental strains, which suggests that the mechanism by which the pathogen acquires nisin resistance may differ among strains. The strains manifested completely unpredicable susceptibility for pediocin without indications for either cross-sensitivity or cross-resistance to nisin and pediocin. Moreover, nisin and pediocin were more effective in a mixture than individually against nisin-sensitive and nisin-resistant *L. monocytogenes* Scott A. In addition it was found that all resistant strains became increasingly sensitive to nisin at pH 5.6 as compared to pH 7.2, which offers promise for the use of nisin in a bacteriocin cocktail or in combination with a low pH.

Voorwoord

Met de voltooiing van dit proefschrift sluit ik een leuke, leerzame èn productieve tijd af die ik niet had willen missen. Diverse personen hebben hierin een bijdrage gehad, die ik vanaf deze plaats wil bedanken voor hulp, steun, advies, samenwerking, of wat dan ook. Een aantal personen verdienen een speciaal woord van dank.

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Annette

1

General introduction

Introduction

The consumers' demand for high quality foods which require a minimum effort of preparation has initiated the introduction of ready-to-eat, convenience foods. Many of these foods contain raw or minimally heated components, and therefore pathogenic bacteria may be present as part of their microflora. The increased popularity of those products throughout the world leads to larger production and consumption volumes and, inherently, to a greater chance for health hazardous situations to occur. Since these perishable food products rely heavily on refrigeration as the main preservation factor, the most prominent hazard to be controlled is the facultative anaerobic, cold tolerant pathogen *Listeria monocytogenes*.

The influence of the food matrix on the potential of this food pathogenic bacterium to grow and survive and the interaction between the pathogen and other microorganisms are important research aspects in designing adequate safety measures. These safety barriers have to comply with the consumers' demands (*i.e.*, natural and fresh like). Barriers currently considered are biopreservation using lactic acid bacteria or the bacteriocins they produce and modified atmosphere storage.

The physiology of *L. monocytogenes* has been an almost completely unexploited research area up to now. The objective of the present research was to gain insight in the physiology of this pathogen, in relation to the food matrix and storage environment as encountered in especially ready-to-eat foods. Aspects considered include the availability of essential growth factors, presence of other microorganisms and presence of protective compounds, thereby paying attention to physico-chemical parameters, like temperature, water activity, pH and oxygen concentration, where appropriate. In addition, research was focussed on the physiological implications of the application of bacteriocins for growth of *L. monocytogenes*.

The literature survey below gives an introduction to *Listeria monocytogenes*, followed by background information on some relevant topics with regard to the content of this thesis, *i.e.*, energy generation and transport mechanisms in bacteria, peptide transport, osmoregulation and bacteriocins. Finally, the structure of this thesis is outlined.

Listeria monocytogenes

History

The first description of the bacterium dates back to 1926, when Murray *et al.* (94) isolated a short, gram-positive, non-sporing, rod-shaped bacterium causing disease in rabbits and guinea pigs. It was named *Bacterium monocytogenes* because it infected the monocytes (white cells) in the blood. Although Murray *et al.* (94) are generally credited with the first accurate description of the causative agent of listeriosis, earlier reports of organisms that caused "listeric-like" infections in humans and animals date back to the late nineteenth century and are comprehensively reviewed by Gray and Killinger (50). In 1927, Pirie isolated the organism from the livers of sick gerbils and called it *Listerella hepatolytica*, after the famous surgeon Joseph Lister. Since the name *Listerella* had previously been adopted for a group of slime moulds, the name *Listeria monocytogenes* was finally agreed (105).

The genus *Listeria*

Listeriae are gram-positive, non-sporing rods, catalase-positive, facultative anaerobic micro-organisms that can grow at temperatures between 0 and 45°C, at pH values between 4.5 and 9, at water activities above 0.92, and in the presence of 10% salt. The genus *Listeria* comprises six species: *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. ivanovii*, *L. welshimeri*, and *L. grayi* (syn. *L. murrayi*) (116, 124). *L. innocua* and *L. grayi* are considered non-pathogenic, while *L. seeligeri*, *L. ivanovii* and *L. welshimeri* rarely cause human infection (28, 64, 82), leaving *L. monocytogenes* as the most important species. The genus *Listeria* is listed in the latest edition of Bergey's Manual of Determinative Bacteriology in group 19 (regular, non-sporing gram-positive rods) together with the genera *Brochotrix*, *Carnobacterium*, *Caryophanon*, *Erysipelothrix*, *Kurthia*, *Lactobacillus*, and *Renibacterium* (55).

Foodborne transmission

Infections due to *L. monocytogenes* have been reported in a wide variety of animals, including cattle, sheep, birds, rodents and fish (50). Contaminated animal feed, especially poorly made silage, was recognized as a cause of listeriosis, but the existence of a similar cause of human infection was not widely appreciated until the early 1980s. It is now generally agreed that the consumption of contaminated foods is the principal route of transmission of listeriosis (139). The consumption of a diverse range of foods has now been reported in the world literature to be associated with transmission of both outbreaks of listeriosis (Table 1) as well as sporadic cases (Table 2).

Because of the ubiquitous nature of *L. monocytogenes*, the presence of the pathogen on raw food items is unavoidable. Recombination of foods which are exposed to the environment or to handling after processing is not always fully controlled. Indeed, *L. monocytogenes* has been isolated from all main categories of food, including milk and dairy products, meat and meat products, vegetables and seafood (39). The foods implicated in listeriosis cases (Table 1 and 2) were generally designed to be consumed without further cooking (*i.e.*, ready-to-eat), with extended shelf lives (8-24 weeks) at refrigeration temperature enabling *L. monocytogenes* to grow (115).

The minimal infective dose of *L. monocytogenes* for humans is not known. The infective dose of foodborne pathogens depends on a number of variables including the conditions of the host, the virulence of the strain, the type and amount of food consumed, and the concentration of the pathogen in the food. Given the ubiquity of the bacterium, and what is known from its distrubution in food and the environment, everybody is exposed to one or more *L. monocytogenes* cells every day, yet the number of illnesses are remarkable low. However, mild forms of *L. monocytogenes* infections may pass unrecognized since they are asyptomatic or at most produce influenzalike symptoms like diarrhoea and abdominal pain that also occur in other cases of gastroenteritis. In the cases described in Table 1 and Table 2, the symptoms were usually more severe, resulting from systemic infections (see also paragraph on pathogenicity).

Table 1. Food-borne outbreaks due to *L. monocytogenes*

Country	Year	No. of cases (deaths)	Food(s) implicated	Reference
Germany	1949-1957	± 100	Raw milk	50
USA	1979	20 (5)	Celery, tomatoes, lettuce[a]	53
New Zealand	1980	29 (9)	Shellfish, raw fish[a]	81
Canada	1981	41 (17)	Coleslaw	122
USA	1983	49 (14)	Pasteurized milk[a]	41
USA	1985	142 (48)	Mexican cheese	83
Schwitzerland	1983-1987	122 (31)	Soft cheese	15
Austria	1986	28 (7)	Raw milk, vegetables[a]	3
USA	1986-1987	36 (16)	Ice cream, salami[a]	123
UK	1987-1989	> 300 (?)	Pâté	90
USA	1989	9 (1)	Shrimps[a]	114
Australia	1990	11 (6)	Pâté	71
France	1992	279 (85)	Jellied pork tongue	117
France	1993	33 (?)	Pork pâté	60
Italy	1993	39 (0)	Rice salad	120
USA	1994	52 (0)	Chocolate milk	30
France	1995	20 (6)	Raw-milk soft cheese	49

[a] indicates an epidemiological association only, without recovery of the implicated strain from the specific food item.

Table 2. Foods implicated in several reports of sporadic cases of food-borne listeriosis (39, 121)

Turkey frankfurters	Soft cheeses	Salted mushrooms
Sausages	Goat cheese	Pickled olives
Undercooked chicken	Other cheeses	Alfalfa tablets
Chicken nuggets	Rennet	Blueberries
Uncooked hotdogs	Human breast milk	Strawberries
Cajun meat	Fresh cream	Nectarines
Fish	Ice cream	
Smoked cod		
Smoked mussels		

Numbers of *Listeria* in contaminated food responsible for epidemic and sporadic foodborne cases are scarce since the incriminated food is rarely available, because of the long incubation periods shown by some patients; the incubation period varies between 1 up to 90 days, with an average of around 30 days (83). In the rare instances where numbers are available, the levels have been generally high ($> 10^3$/g) (61). Although it is likely that the infective dose for foodborne listeriosis is fairly high, caution is needed because of the small number of cases where information is available and the likelihood of great differences in susceptibility to infection between individuals (115).

Detection methods in foods

Several countries have adopted absence of *L. monocytogenes* cells in 0.01 gram of product, whereas food laws of other countries have imposed a zero tolerance for *L. monocytogenes* in foods, which translates to less than one cell in 25 gram. Therefore, in most instances, an enrichment step is required for a succesful isolation of the pathogen. For many years, cold enrichment followed by isolation on selective or non-selective media was applied (51, 136). When the significance of *L. monocytogenes* was appreciated, cold enrichment techniques were found too time-consuming and cumbersome for routine use and therefore various selective enrichment and plating procedures were developed (36). To date effective methods are available both for the isolation and detection of *Listeria*.

The conventional detection methods have been partly replaced by so-called rapid methods, these methods use immunological or genetic techniques for the detection of *Listeria* spp. in general or for *L. monocytogenes* in particular. Latex tests, enzyme-linked immunosorbent assays (ELISA's) and DNA-probes are commercially available (54). PCR-based detection methods have also been developed, but none of them has appeared on the market yet. Interference of the food matrix in the performance of nucleic acid amplification reactions is the major hurdle to be taken before a successful introduction of these methods in routine microbiological analysis of food can be expected (79).

Recently, it has been demonstrated that the incidence of *L. monocytogenes* may be underestimated, because other listerias may mask the presence of the pathogen (11, 87, 103). For this reason, protocols for the detection of *L. monocytogenes* should include an isolation medium which allows identification of *L. monocytogenes* in presence of high numbers of other listeriae (12). Enhanced haemolysis agar (EHA), which distinguishes *L. monocytogenes* from non-pathogenic listeria on basis of hemolysis (12, 26) is suitable, or differentation on the basis of lecithin degradation in *L. monocytogenes*, which is not evident in the other five *Listeria* species may be exploited (23). Other differentation methods include the CAMP test, which depends on the synergistic lysis of erythrocytes by *L. monocytogenes* in the presence of extracellular enzymes produced by *Staphylococcus aureus* (88) and a specific API system (16).

Pathogenicity

Listeriosis is not characterized by a unique set of symptoms because the course of the

disease depends on the state of the host. Almost all *L. monocytogenes* infections occur because of a predisposing immunologic deficiency. People with a higher risk for listeriosis than the normal population include pregnant women and, patients with AIDS, malignancy undergoing chemotherapy, neoplasm, diabetis, alcoholism, chronic liver disease, renal disease, cardiovascular disease etc. In cases not involving pregnancy, the syndromes mainly include meningitis with or without bacteraemia. Other syndromes include endophthalmitis, septic arthritis, osteomyelitis, pericarditis, endocarditis, cholecystitis, pneumonia, peritonitis, brain and liver abscesses. Pregnant females who contract the disease present influenzalike symptoms or may not present any symptoms at all. However, abortion, premature birth, or stillbirth is often the consequence of listeriosis in pregnant females (39, 121).

The crucial steps in the interaction of *L. monocytogenes* with eukaryotic host cells have been extensively studied in recent years using epithelia-like and macrophage-like cells (reviewed in references 76 and 125). The host cell infection is mediated by internalin, a protein encoded by the gene *inlA*, and begins with the internalization of the bacteria by phagocytosis, in the case of macrophages, or by induced phagocytosis in normally nonphagocytic cells. Subsequent to internalization, a significant proportion of invading bacteria escape from a vacuole, multiply, and are propelled through the cytoplasm by inducing the polymerization of host actin. Bacteria spread from cell to cell by inducing formation of pseudopodlike structures, each containing a bacterium at its tip, which are subsequently internalized by neighbouring cells. At this stage, bacteria are found in double-membrane vacuoles from which they can escape to repeat the cycle. By this process, *L. monocytogenes* gains access to target organs (*i.e.*, placenta and central nervous system including the brain).

The virulence factors are encoded by a chromosomal gene cluster consisting of six genes in the order *prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB*. These genes encode two phospholipases of the C type (PLC), specific for phosphatidylinositol (PI-PLC) (*plcA*) and phosphatidylcholine (PC-PLC or lecithinase) (*plcB*), and a hemolysin called listeriolysin O (*hly*) which have all been implicated in vacuole lysis. A metalloprotease (*mpl*) is responsible for the processing of PC-PLC and *actA* codes for a protein that is necessary for induction of actin polymerization. This gene cluster and additional genes including the *inlA* gene, which are not part of the cluster, are under control of the transcriptional activator PrfA. High temperatures, high salt concentrations and nutrient stress lead to the expression of the PrfA protein (23, 80, 128).

Nutritional requirements and metabolism

Listeriae grow well in many media such as brain heart infusion, trypticase soy, and tryptose broths. Most nutritional requirements have been described for *L. monocytogenes*, the other species are believed to be similar. Magnesium and phosphate are necessary, while potassium is at least growth stimulatory. Four vitamins (biotin, riboflavin, thiamin, and thioctic acid) and five amino acids (cysteine, methionine, valine, leucine, isoleucine) are required (13, 112). These amino acids cannot be acquired through hydrolysis of milk or

proteins such as casein or gelatin since *L. monocytogenes* is non-proteolytic (124). Iron is an essential nutrient for *L. monocytogenes*, a surface-associated ferric reductase system has been suggested to be involved in iron acquisition rather than siderophores (34).

L. monocytogenes depends on carbohydrates for its primary energy source, glucose is reported to be its preferred carbohydrate (50, 91, 104, 112, 124). Recently, it was evidenced that *L. monocytogenes* possesses two glucose transport systems, a high-affinity phosphoenolpyruvate dependent phosphotransferase system (PTS) and a low-affinity proton motive force (PMF)-mediated system (96). Glucose is utilized by the Emden-Meyerhof pathway, there is no evidence for the Entner Doudoroff pathway and the pentose phosphate pathway has also been excluded as an important means for glucose metabolism. Anaerobically, the end product is mainly lactic acid; aerobically lactic acid, acetic acid and some acetoin (and concomitantly CO_2) are produced (91, 104, 118). Small amounts of isobutyric and isovaleric acid have also been detected under aerobic conditions, but these compounds are thought to be derived from oxidation of isoleucine and leucine, respectively (104).

L. monocytogenes has been shown to lack a complete tricarboxylic acid cycle due to the absence of the enzymes α-ketoglutarate dehydrogenase and succinate dehydrogenase, whereas the enzymes isocitrate lyase and malate synthase of the glyoxylate bypass are also absent (132). Although most citrate cycle enzymes are present in *L. monocytogenes*, the pathogen is not able to oxidize or to grow on citrate, isocitrate, α-ketoglutarate, succinate, fumarate, malate, acetate or pyruvate. This is most likely due to the lack of the requisite uptake systems (43, 104, 132). Due to the noncyclic split citrate pathway, the capacity to generate ATP and reduced NAD(P)H is limited. It has therefore been suggested that in *L. monocytogenes* this pathway is important for biosynthesis (*i.e.* glutamate and aspartate) and not for a net gain in energy (5, 132).

Information about electron transfer chain activity in *L. monocytogenes* is contradictory. Two reports demonstrate the presence of cytochromes (40, 44), whereas cytochromes could not be detected by Trivett and Meyer (132). Patchett *et al.* (98) also failed to detect cytochromes in *L. monocytogenes*, but found oxidase activity associated with the cell membrane, which was inhibited by classical inhibitors of the electron transfer chain (cyanide, rotenone, 2-heptyl-4-hydroxyquinoline-*N*-oxide). Taken together, these data seem to indicate a low concentration of respiratory compounds in *L. monocytogenes*, which is justified by the low production of NAD(P)H via the noncyclic citrate pathway. Recently, Houtsma (56) reported that the oxygen consumption rate in *L. innocua* cells grown without shaking was 200 nmol O_2 min^{-1} mg of $protein^{-1}$. Cyanide (10 mM) appeared to have a moderate inhibitory effect on oxygen consumption and therefore it was suggested that *L. innocua* might possess a cytoplasmic NADH-oxidase in addition to low concentrations of cytochrome oxidases. In the presence of cyanide *L. innocua* was able to build up a transmembrane pH gradient upon addition of glucose which argues against an important role of a proton-translocating electron transport chain in PMF generation in *L. innocua* (56). The addition of *N,N'*-dicyclohexylcarbodiimide (DCCD) to *L. monocytogenes* cells in which the pH gradient was collapsed by nigericin, has been demonstrated to result in a complete dissipation of the

membrane potential (134, 135), which also suggests that a PMF is merely formed by a proton translocating ATPase in *Listeria* spp.

In lactic acid bacteria under aerobic conditions, a cytoplasmic NADH-oxidase serves as a means of regenerating NAD^+ needed for glycolysis (25, 126) by the conversion of lactate to acetate. Replenishment of NAD^+ under anaerobic conditions is achieved via conversion of pyruvate to lactate. This oxidase allows the bacteria to form additional ATP by substrate level phosphorylation in the presence of oxygen. Listeriae may follow a similar strategy to increase fermentation yield under aerobic conditions since growth of *Listeria* spp. in the absence of oxygen yields lactate as the major metabolite, whilst under aerobic conditions both lactate and acetate are produced. Moreover, acetate kinase and phosphate acetyltransferase activities have recently been detected in *L. innocua* cells grown aerobically (67). The oxidation of NADH might be carried out by a cytoplasmic NADH-oxidase or via an electron transfer chain.

Bioenergetics and solute transport in bacteria

In bacteria two forms of metabolic energy are usually present: energy-rich phosphate bond intermediates with ATP as the major representative and electrochemical energy stored in ion gradients, mainly proton and/or sodium gradients. These two forms of metabolic energy can be obtained by substrate level phosphorylation, in which the free energy of a particular enzyme reaction is directly used for the synthesis of ATP, and by chemiosmotic free energy-conserving processes, in which light or redox energy is converted into electrochemical energy by coupling the chemical reactions to the translocation of ions across the membrane (75, 92). The electron transfer systems (aerobic and anaerobic electron transfer systems, phototrophic light-driven electron transfer systems) and the membrane-bound F_0F_1-ATPases are such proton and/or sodium translocating systems. These energy converters are termed primary transport systems (Fig.1). The electrochemical gradients of the translocated ions exert a force on these ions, the ion motive force. If protons are the translocated species a proton motive force (PMF) is formed. The PMF is composed of two components: an electrical potential ($\Delta\psi$), and a chemical potential of protons ($-Z\Delta pH$; $Z = 2.3\ RT/F$) across the membrane. In equation: $PMF = \Delta\psi - (2.3RT/F)\Delta pH$ (expressed in mV). In case of a sodium motive force, ΔpH is substituted by ΔpNa.

Other translocators that belong to the class of primary transport systems include binding protein-dependent transport systems and export proteins (ATP binding cassette (ABC) transporters). Binding protein-dependent transporters are multisubunit systems consisting of a periplasmic binding protein, that is free in the periplasmic space in gram-negative bacteria and anchored to the cytoplasmic membrane in gram-positive bacteria, and further often two hydrophobic membrane proteins, and two ATP-binding subunits (6, 45). Transport requires the hydrolysis of ATP and is blocked by vanadate. These systems function in the uptake of various solutes (amino acids, sugars, peptides, (in)organic ions, vitamins, osmolytes) (for details see Poolman *et al.*, (109) and references herein).

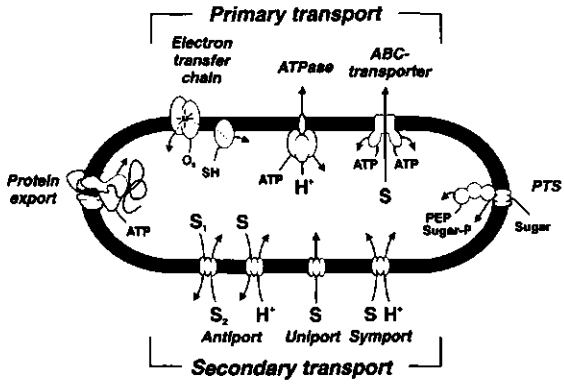


Figure 1. Schematic presentation of transport systems in the cytoplasmic membranes of bacteria. (adapted from reference 75).

In secondary transport systems the energy for translocation of one solute is supplied by (electro)chemical gradients of other solutes (including ions). Secondary transport systems are comprehensively reviewed by Poolman and Konings (108). Three general categories of these transporters can be distinguished in bacteria (Fig. 1). The transport is indicated uniport when the transport is independent of any coupling ion. When two or more solutes move in the same direction, the transport process is called symport, or cotransport. In this type of transport the (electro)chemical gradient of one solute (usually proton(s) or sodium ion(s)) is used to drive the uphill transport of the other solute. Antiport or countertransport refers to the movements of solutes in opposing directions. Since the solutes transported by secondary transport systems can be neutral or negatively or positively charged and different numbers of solutes may be co- or countertransported, the driving forces of these processes may differ considerably. Secondary transport systems generally exhibit a much lower affinity for the substrate as compared to binding protein-dependent transport systems. Secondary transporters mediate the transport of various solutes including amino acids, sugars, peptides and (in)organic ions (108).

Recently, Jacobs *et al.* (59) demonstrated that *Rhodobacter sphaeroides* contains a Na^+-stimulated glutamate transport system that is driven by the PMF, but strictly requires the presence of a periplasmic glutamate binding protein. All binding protein-dependent transport systems described so far belong to the family of ATP binding cassette proteins.

The activity of secondary transport systems can lead to the generation of a PMF, which, subsequently, can drive other metabolic energy-requiring processes such as ATP synthesis via the membrane bound F_0F_1-ATPase. These processes are rather common in anaerobic and fermentative bacteria and play an important role in the bioenergetics of these organisms. Product excretion as well as substrate or precursor uptake can contribute to energy conservation in the form of an ion motive force. If the overall transport process is

electronegative, *i.e.* a net negative charge is moved inwards or net positive charge is moved outwards against the $\Delta\psi$ (inside negative), the electrical potential will increase. Moreover, if in the overall transport process protons or sodium ions are translocated against the respective concentration gradients, the ΔpH or ΔpNa will increase. Finally, if in the metabolism of the accumulated solute (precursor) net protons are consumed, a ΔpH is obtained. Various examples of generation of metabolic energy by secondary transport processes, particularly in lactic acid bacteria, are given in Poolman (107) and in Konings *et al.* (74, 75).

The third type of uptake mechanism is group translocation. Group translocation systems couple the translocation of a solute to the chemical modification of the solute resulting in the release of a modified solute at the other side of the membrane. The only group translocation systems found in bacteria are the phosphoenolpyruvate:sugar phosphotransferase systems (PTS). These systems catalyze the concomitant uptake and phosphorylation of sugars in an overall irreversible reaction (Fig. 1). Details on PTS are reviewed by Postma *et al.* (110).

Peptide transport

Peptide transport has been well documentated in bacteria, yeast, fungi, plants and mammals (86, 101). In bacteria, these peptides are, after internalization, hydrolysed to serve as sources of amino acids and nitrogen. In addition to acquiring nutrients from the environment, peptide transport has been shown to play a role in recycling cell wall peptides, osmoregulation, in transducing signals for induction of sporulation, chemotaxis, and in synthesis of virulence factors (7, 29, 48, 68, 84, 85, 89, 97, 102).

The understanding of the physiological importance, structures and properties of bacterial peptide transport systems originates from studies with the enteric gram-negative bacteria *Escherichia coli* and *Salmonella typhimurium*. These bacteria possess three distinct peptide transport systems (reviewed in reference 101) with overlapping substrate specificities. The dipeptide permease (Dpp) transports mainly dipeptides but has in addition some affinity for a number of tripeptides. The second system is the tripeptide permease (Tpp) which transports di- and tripeptides containing hydrophobic amino acid residues. Finally, the oligopeptide permease (Opp) transports essentially any peptide containing up to six amino acid residues, more or less irrespective of its amino acid composition. All three peptide transporters belong to the ATP binding cassette family of transporters.

Bacteria other than *E. coli* and *S. typhimurium* that have been studied extensively regarding peptide transport involve the lactic acid bacteria, particularly lactococci. The proteolytic system of these bacteria has been studied in detail (reviewed in reference 77), mainly because of their commercial importance in casein breakdown during cheese production. The presence of two di- and tripeptide systems and one oligopeptide transport system, as observed in the enteric bacteria, is also observed in *L. lactis*. The two di-tripeptide carriers (DtpT and DtpP) have partial overlapping substrate specificities. DtpP transports preferentially di- and tripeptides that are composed of hydrophobic residues, whereas DtpT has a higher specificity for more hydrophilic and charged peptides (42). The

oligopeptide transport system (Opp) of *L. lactis* mediates the transport of peptides of four to at least eight residues. Transport of peptides in *L. lactis* via DtpP and Opp is driven by ATP, however, DtpT is unique among bacterial peptide transporters, as it is encoded by a single gene and uses the PMF to drive peptide transport. DtpT belongs to the PTR family of peptide transporters, members of this family were hitherto only identified in eukaryotes. This family is characterized by several conserved motifs, has 12 putative transmembrane domains, and is driven by the PMF (52, 129).

Peptide transport has also received attention in some other gram-positive bacteria. *Bacillus subtilis* possesses three ABC-type permeases. For the translocation of dipeptides it expresses a dipeptide permease (Dpp) (85), while it has two oligopeptide transport systems for the uptake of tri-, tetra, and pentapeptides, which are designated Opp and App (73, 102). *Streptococcus pneumoniae* incorporates peptides of two to seven amino acid residues via a binding protein-dependent permease (Ami) (4). A Hpp system in *Streptococcus gordonii*, which is genetically and structurally similar to the Ami permease, transports primarily hexa- and heptapeptides (62).

Peptide transport systems have also been reported to exist in other bacterial species including the gram-negative species *Pseudomonas aeruginosa, Bacteriodes ruminicola, Streptococcus bovis* and the gram-positive species *Staphylococcus aureus, Enterococcus faecalis* and *Mycoplasma hyorhinis* (see reference 101 for details).

Osmoregulation

Increased osmotic pressure is one of the oldest food preservation techniques which is still in use. Desiccation or addition of high amounts of osmotically active substances, such as salt or sugars lower the water activity (a_w) of the food. Understanding of the processes underlying osmotic adaptation in pathogens like *L. monocytogenes*, is of crucial importance in trying to control their growth in low and medium a_w foods.

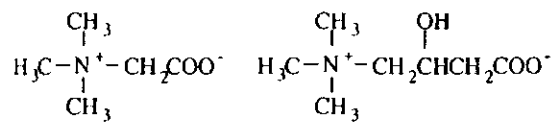
Bacteria maintain an osmotic pressure in the cytoplasm that is higher than that of the surrounding environment, which results in a pressure exerted outwards on the cell wall, the turgor. In response to a hyperosmotic shock, bacteria have evolved special mechanisms for controlled intracellular accumulation of solutes in order to restore turgor. These so-called compatible solutes are not inhibitory to most cellular processes and may even stabilize enzymes and membranes (18, 106, 119, 140).

The physiology and genetics of bacterial responses to osmotic stress have been most extensively studied with the gram-negative species *E. coli* and *S. typhimurium* (reviewed in reference 27). The primary response towards an increase in medium osmolarity in these bacteria involves the uptake of potassium and synthesis (or transport from the environment) of glutamate. Another early response includes the uptake of proline and betaine via activation of the constitutive ProP carrier, which is a sodium-dependent uptake system. Following expression of the inducible binding protein-dependent ProU system, accumulation levels of proline and betaine are subsequently increased further. *E. coli* is able to synthesize betaine from exogenously provided choline (78); *S. typhimurium* lacks the necessary genes and

therefore is unable to use choline as an osmoprotectant. In the absence of betaine, *E. coli* synthesizes the disaccharide trehalose.

The situation in gram-positive bacteria has gained considerable less attention in the past, but recently the research interest in osmoregulation within these bacteria has revived. Research in this area has been mainly focussed on *B. subtilis*, *Staphylococcus aureus*, *L. monocytogenes* and some lactic acid bacteria. A role for potassium has been demonstrated (47, 99, 138), whereas the intracellular pools of several amino acids (proline, glutamate, glutamine, asparagine, aspartate, glycine and alanine), were shown to increase either via synthesis or transport from the exterior in gram-positive bacteria (47, 63, 70, 93, 99, 137). Specific transport systems for betaine and/or proline have been detected in gram-positive species. *S. aureus* possesses a high-affinity transport system for proline and a high-affinity transport system for betaine and an uptake system which shows low affinity for both proline and betaine, which function as secondary carriers using sodium as co-substrate. (8, 9, 111, 131). Three different transport systems for betaine operate in *B. subtilis* designated OpuA, OpuC and OpuD, all belong to the ATP binding cassette uptake systems (66). *B. subtilis* is able, like *E. coli*, to convert choline into betaine (17). Transport of betaine and/or proline has been studied in three species of lactic acid bacteria: *Lactobacillus acidophilus, Lactobacillus plantarum,* and *Lactococcus lactis* (46, 58, 63, 93). From these studies it can be deduced that these bacteria have distinct transport systems for proline and betaine. The transport of betaine proceeds via a specific high-affinity transporter, whereas the proline transporter generally shows in addition some affinity for betaine. Betaine transport in *L. plantarum* requires ATP (46), the energy sources for translocation of betaine and proline in *L. lactis* and *L. acidophilus* remain unclear (58, 63, 93). In *L. monocytogenes*, betaine is transported via a high-affinity secondary transport protein, which shows no affinity for proline (44, 100).

Recently, a crucial role of carnitine was shown in the osmoregulation of *L. monocytogenes* (13, 134) and *L. plantarum* (69). A great value is attached to this compatible solute in the osmoregulation of food associated microorganisms, since this compound is ubiquitous in biological material due to its crucial role in the ß-oxidation in mitochondria. Carnitine is consequently abundant especially in food products of animal origin (Table 3) (14, 24, 127). Betaine is a plant metabolite and is therefore in particular present in food products of plant origin (10, 113). However, the presence of betaine has also been demonstrated in some meat products (127). Structural formulas of betaine and carnitine are depicted in Figure 2.



Betaine (Glycinebetaine) **L-Carnitine**

Figure 2. Molecular structures of betaine and carnitine

Table 3. Carnitine content of some food products (24, 127)

plant derived	mg/100 g	*animal origin*	mg/100g
avocado	1.25	beef	59.8-67.4
cabbage	-[a]	beef kidney	1.8
cauliflower	0.13	processed meats	4.6-19
orange juice	-	lamb	78.0
peanut	0.76	chicken	4.6-9.1
spinach	-	egg	-
bread	0.24	cow's milk	0.53-3.91

[a] = not detected

Bacteriocins

Bacteriocins are small antibacterial proteins produced by many bacteria. Those produced by lactic acid bacteria have been extensively studied for use as food preservatives which is mainly based on the GRAS (generally regarded as safe) status of LAB (reviewed in reference 130). The major classes of bacteriocins produced by lactic acid bacteria include (I) small, posttranscriptionally modified lantibiotics; (II) small (< 10 kDa), heat-stable unmodified peptides, subdivided into *Listeria*-active peptides with the N-terminal consensus sequence -Tyr-Gly-Asn-Gly-Val- (class IIa), poration complexes requiring two different peptides for activity (class IIb), and thiol-activated peptides requiring reduced cysteine residues for activity (class IIc); (III) large (> 30 kDa) heat-labile proteins, and (IV) complex proteins whose activity requires the association of carbohydrate or lipid moieties (72). The antibacterial activity of most bacteriocins is directed against species that are closely related to the producer and also against different strains of the same species as the producer. The relevance of bacteriocins to food microbiology is that there are bacteriocins that are active against a number of other gram-positive bacteria causing spoilage and foodborne diseases. Gram-negative bacteria are generally not sensitive to broad-spectrum bacteriocins, like nisin, since the porins in the outer membrane of gram-negative bacteria are too small to allow free passage of these compounds.

The only bacteriocin produced commercially with a legal status to be used as a food additive is nisin (31). Nisin is a 34-residue antibacterial peptide that is produced by several strains of *L. lactis* and belongs to class I bacteriocins. The inhibitory effect of nisin is broader than that of most other bacteriocins and extends to a wide variety of gram-positive bacteria, including *L. monocytogenes* and sporefomers. Recent reviews of nisin include those of Hurst and Hoover (57), De Vuyst and Vandamme (35), Delves-Brougton and Gasson (33) and Delves-Brougton *et al.* (32). Another interesting bacteriocin with regard to biopreservation of foods is pediocin PA-1, which is produced by *Pediococcus acidilactici*. Pediocin PA-1, a 44-residue peptide, constitutes the best-characterized class II bacteriocin

(22). It belongs to the so-called antilisterial or pediocin-like peptides (subgroup IIa).

Class I and II bacteriocins are believed to form pores in the membrane of target cells. This pore formation causes a rapid efflux of small cytoplasmic molecules and ions from the target cells and a collapse of the PMF, which may lead to cell death (1, 2, 19, 20, 22). Pore formation by nisin and other lantibiotics requires a threshold potential (37, 65). At least two models may explain the membrane-permeabilization action of nisin (Fig. 3). The first model, suggests that nisin monomers contact the membrane, insert in the presence of a $\Delta\psi$, and subsequently the different monomers oligomerize in the cytoplasmic membrane to form a pore (95). An alternative model suggests that several nisin molecules bind parallel to the anionic phospholipids of the membrane, previous to a $\Delta\psi$ dependent insertion of nisin molecules into the membrane (37, 133). Pediocin PA-1 is believed to require a membrane-associated protein receptor and to act in a voltage-independent way (19, 22). However, pediocin was recently shown to cause efflux of carboxyfluorescein from lipid vesicles in absence of a protein receptor (21).

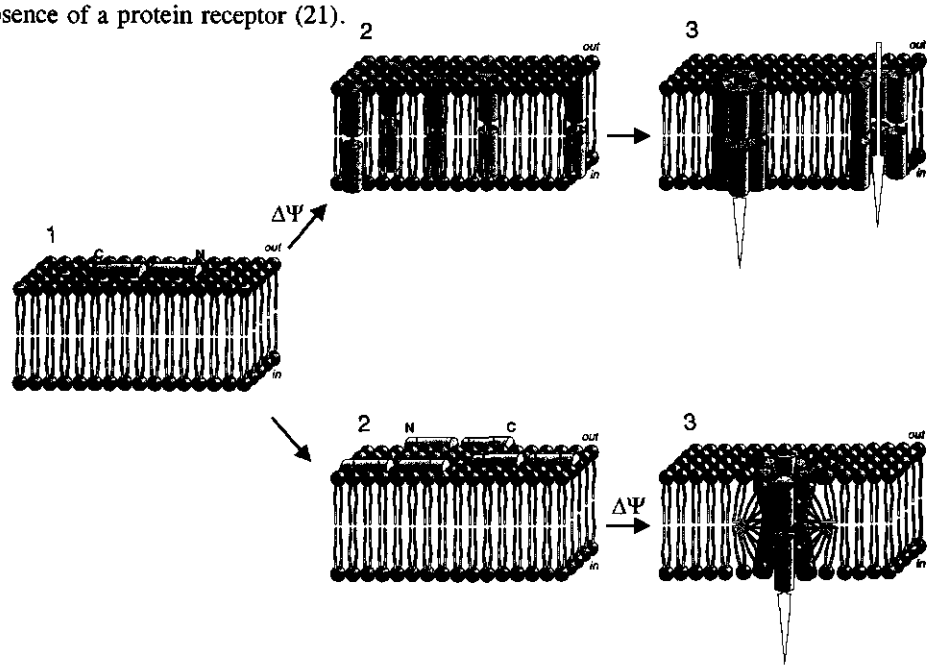


Figure 3. Models for pore formation of nisin. The mature nisin molecule is schematically presented with the *N*-terminal (N) 1-19 amino acid residue part containing two lysine residues, which are positively charged, connected via a flexible hinge region to the 21-34 terminal (C) amino acid residue part, which contains one lysine residue. The light and dark shaded halves represent, respectively, the hydrophilic and hydrophobic regions of the amphilic peptide. The first mechanism (*top*) involves three discrete steps: *1.* binding of nisin molecules to the membrane, *2.* $\Delta\psi$ (inside negative)-dependent insertion into the membrane, and *3.* aggregation of monomers resulting in the formation of a water-filled pore. In the second model (*bottom*), the nisin monomers are assumed to associate at the surface of the membrane, followed by $\Delta\psi$ (inside negative)-dependent insertion into the membrane with the (anionic) phospholipid headgroups incorporated into the pore (adapted from reference 1).

Thesis outline

This thesis deals with the physiology of the important psychrotrophic foodborne pathogen *L. monocytogenes*. The physiology of the pathogen was studied in connection with peptide utilization, osmoregulation and the resistance to bacteriocins.

Chapters 2 and 3 give a detailed description of the utilization of peptides by *L. monocytogenes*. It is demonstrated that di- and tripeptides are translocated into the cell via a PMF-driven transport system and subsequently hydrolyzed in the cytoplasm (Chapter 2). An ATP-dependent oligopeptide transporter in combination with intracellular peptidases allows efficient utilization of larger peptides composed of up to eight amino acid residues (Chapter 3). *L. monocytogenes* is non-proteolytic and multiple amino acid auxotroph which emphasizes the relevance of these findings. Attention is paid to the significance of the results for the understanding of enhanced growth of the pathogen in certain foods by the presence of proteolytic microorganisms and/or enzymes.

In Chapter 4 the role of amino acids and peptides in the growth of *L. monocytogenes* at high osmolarity is presented. The ability to tolerate environments with elevated osmolarity is an important factor in the pervasiveness of *L. monocytogenes*. In addition to their role in nitrogen metabolism, peptides can be accumulated in the cytoplasm by uptake from the external medium thereby conferring osmoprotection in *L. monocytogenes*. This is a novel finding since, although peptide accumulation during osmoregulation has previously been noted, such peptides are usually *de novo* synthesized, rather than accumulated from the external medium.

Other compounds that have been implicated in osmoregulation in *L. monocytogenes* involve betaine and L-carnitine. In Chapter 5 the transport of L-carnitine in *L. monocytogenes* is described in detail. L-carnitine transport appears to be mediated by a highly specific ATP-dependent transport system with a high scavenging capacity for its substrate. The results from this study, initiated a study on the regulation of betaine and L-carnitine transport in *L. monocytogenes*, which is reported in Chapter 6. It is evidenced that betaine and L-carnitine enter the cytoplasm solely via distinct transporters in *L. monocytogenes* and a new osmolyte sensing mechanism is presented.

In Chapter 7 the role of carnitine in the osmotic stress response of the gram-negative *E. coli* is described with a focus on enterohemorrhagic *E. coli* 0157:H7. The ability of *E. coli* to import L-carnitine via the general osmoporters ProP and ProU is presented. Furthermore, enterobacteriaceae like *E. coli* are known to convert carnitine, via crotonobetaine, to γ-butyrobetaine in the presence of C- and N-sources under anaerobic conditions. This pathway includes CaiT, a carrier-mediated uptake system (38). Therefore, the possible involvement of CaiT during growth at high osmolarity under anaerobic conditions was also assessed.

The introduction of bacteriocins as an extra safety hurdle in ready-to-eat foods introduces the emergence of bacteriocin-resistant *L. monocytogenes*. Insight in the mechanism of bacteriocin-resistance will provide a rational basis for bacteriocin application in foods. Chapter 8 deals with the mechanism of nisin-resistance in a strain of *L. monocytogenes*. The membrane composition of a nisin-resistant mutant *L. monocytogenes* strain is compared with

that of its parent strain and the capability of both strains to synthesize phospholipids during nisin exposure is described. Furthermore, monolayer studies supplied information on specific interactions of nisin with the lipids of both strains. These approaches provide important new information on the role of membranes in nisin resistance. Further characterization of the nisin-resistance phenomenon in *L. monocytogenes* is presented in Chapter 9. A range of nisin-resistant *L. monocytogenes* strains and their wild-type strains were tested for their sensitivity towards mutanolysin, pediocin and nisin. Moreover, growth characteristics of the strains at refrigeration temperature (7°C) and optimal growth temperature (30°C) are described and attention is paid to the application of both bacteriocins in milk in order to inhibit *L. monocytogenes* and possible nisin-resistant variants.

Finally, the importance of these findings in relation to growth and survival of *L. monocytogenes* under stress conditions are integrated and discussed in Chapter 10.

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2

A di- and tripeptide transport system can supply *Listeria monocytogenes* Scott A with amino acids essential for growth

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Abstract

Listeria monocytogenes takes up di- and tripeptides via a proton motive force-dependent carrier protein. This peptide transport system resembles the recently cloned and sequenced secondary di- and tripeptide transport system of *Lactococcus lactis* (A. Hagting, E.R.S. Kunji, K.J. Leenhouts, B. Poolman, and W.N. Konings. J. Biol. Chem. 269: 11391-11399, 1994). The peptide permease of *L. monocytogenes* has a broad substrate specificity and allows transport of the nonpeptide substrate 5-aminolevulinic acid, the toxic di- and tripeptide analogs, alanyl-ß-chloroalanine and alanyl-alanyl-ß-chloroalanine, and various di- and tripeptides. No extracellular peptide hydrolysis was detected, indicating that peptides are hydrolyzed after being transported into the cell. Indeed, peptidase activities in response to various synthetic substrates were detected in cell extracts obtained from *L. monocytogenes* cells grown in brain heart infusion broth or defined medium. The di- and tripeptide permease can supply *L. monocytogenes* with essential amino acids for growth and might contribute to growth of this pathogen in various foods where peptides are supplied by proteolytic activity of other microorganisms present in these foods. Possible roles of this di- and tripeptide transport system in the osmoregulation and virulence of *L. monocytogenes* are discussed.

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Introduction

The fact that food-borne transmission of *Listeria monocytogenes* plays an important role in the etiology of human listeriosis has led to the need for information on the characteristics of this gram-positive psychrotrophic bacterium with regard to foods. *L. monocytogenes* is a common contaminant of raw and minimally processed foods, mainly because of its ubiquity in the environment. Other crucial factors in the upswing of *L. monocytogenes* as a food-borne pathogen are its abilities to grow at refrigeration temperature and in osmotically stressful environments (12). On the other hand, *L. monocytogenes* has a limited biosynthetic capacity as judged by its complex nutritional requirements. Whereas most bacteria are able to synthesize all 20 amino acids necessary for protein biosynthesis by utilizing inorganic ammonium salts as a nitrogen source (5), *L. monocytogenes* demands, in addition to a sugar, vitamins, and iron, five amino acids for growth (7, 33).

To date, no proteolytic activity has ever been reported for the genus *Listeria*. Therefore, *L. monocytogenes* cannot satisfy its amino acid requirement with milk or proteins such as casein and gelatin (37). However, one of the virulence genes (*mpl*) in *L. monocytogenes* encodes a zinc-dependent metalloprotease (Mpl) that is located immediately downstream of the *hly* gene encoding the cytolytic toxin listeriolysin (26). Mpl shows no activity under standard laboratory conditions because of a lack of processing of the inactive pro-form of the protease to its mature active form (9). Mpl was shown recently to be involved in processing phosphatidylcholine-specific phospholipase C (PlcB) from the precursor to its active form (32, 34). PlcB appears to be involved in the disruption of the double membrane which is formed around the bacterium when it enters a neighboring mammalian cell (48). This is consistent with the proposed role of Mpl in the virulence of *L. monocytogenes*, and indeed no homologous sequences have been detected in the nonpathogenic species *Listeria ivanovii*, *Listeria seeligeri*, and *Listeria innocua*. It was therefore suggested that Mpl does not play a role in persistence or growth of *L. monocytogenes* in food environments (9, 24).

Outbreaks of listeriosis have been traced mainly to the consumption of dairy products, meat, and raw vegetables (12, 19). The free amino acid levels in milk are well below the minimum requirement for growth to high cell densities (44), whereas the amount of free amino acids essential to *L. monocytogenes* in meat and vegetables is generally also inadequate (20, 35). Therefore, the supply of essential amino acids for growth of *L. monocytogenes* in these products has to originate from other sources, possibly by degradation of proteins by other microorganisms. Since a mixture of peptides and amino acids becomes available as a consequence of proteolytic breakdown of proteins, it is worthwhile to determine the nutritional value of peptides for growth of *L. monocytogenes*.

Different routes by which exogenous peptides can supply amino acids for growth may be envisaged. The intact peptide can be hydrolyzed on the outside of the cell, after which the liberated free amino acids are taken up by specific amino acid transport systems. Alternatively, the peptide can be translocated by a specific peptide transport system into the cell, where it is subsequently hydrolyzed by intracellular peptidases. Finally, it is conceivable that both peptide transport and hydrolysis are functions associated with the cytoplasmic

membrane (28, 40).

Information about peptide transport in *L. monocytogenes* is presently lacking. For a better understanding of the ability of *L. monocytogenes* to colonize different food products, details on peptide utilization, including peptide transport and hydrolysis, are required. Accordingly, the objective of the current study was to assess the role of dipeptides as the source of essential amino acids for growth of *L. monocytogenes*. The results demonstrate that *L. monocytogenes* can utilize dipeptides to satisfy its nutrient requirements, and evidence is presented that this pathogen possesses a constitutive, proton motive force (PMF)-dependent di- and tripeptide transport system with a broad substrate specificity. Possible roles of this peptide transport system in osmoregulation and virulence of *L. monocytogenes* are discussed.

Materials and Methods

Organism. *L. monocytogenes* Scott A was used throughout the study. The organism was routinely maintained in brain heart infusion broth (BHI) (Gibco) containing 20% (vol/vol) glycerol and stored at -20°C.

Media and growth conditions. Cells were grown at 30 °C in BHI or in a chemically defined minimal medium (DM) described previously by Premaratne *et al.* (33) containing (liter^{-1}): KH_2PO_4, 6.56 g; $Na_2HPO_4.7H_2O$, 30.96 g; $MgSO_4.7H_2O$, 0.41 g; ferric citrate, 0.088 g; glucose, 10 g; L-leucine, 0.1 g; L-isoleucine, 0.1 g; L-valine, 0.1 g; L-methionine, 0.1 g; L-arginine, 0.1 g; L-cysteine, 0.1 g; L-glutamine, 0.6 g; riboflavine, 0.5 mg; thiamine, 1.0 mg; biotin, 0.5 mg; thioctic acid, 0.005 mg.

Dipeptides, tripeptides, ß-chloroalanine, alanyl-ß-chloroalanine, alanyl-alanyl-ß-chloroalanine, and 5-aminolevulinic acid (ALA) were added to DM as required from filter-sterilized stock solutions.

Growth experiments. An overnight culture grown in BHI was subcultured twice in DM before the experimental cultures were inoculated with about 10^4 to 10^5 cells per ml. Aliquots of 200 µl of the cultures were dispensed in the different wells of 96-well sterile tissue culture plates with flat bottoms (Flow Laboratories, Irvine, Scotland). The plates were covered and statically incubated at 30°C. Evaporation of water from the wells was prevented by filling the outer wells with sterile distilled water. Growth was measured at an optical density at 550 nm (OD_{550}) in a microtitre plate reader (Easy Reader 400 FW; SLT-Instruments, Austria), and the OD values were normalized to a path length of 1 cm.

Plate test for determination of toxic effects of chlorinated compounds. The toxicities of the chlorinated amino acid (ß-chloroalanine) and peptide analogs (alanyl-ß-chloroalanine and alanyl-alanyl-ß-chloroalanine) were tested by plating approximately 10^8 CFU on a 1.5% (wt/vol) agar plate containing DM. Single crystals of one of the toxic analogs were put in the center of the plate. The growth inhibition, indicated by the presence of a clear zone, was determined after 4 days of incubation at 30°C.

Preparation of cell free extract (CE) and determination of peptidase activity. Cells of *L. monocytogenes* were harvested during logarithmic growth at an OD_{600} of 0.6, washed twice in 50 mM potassium phosphate (pH 6.9), and resuspended in this buffer to a final OD_{600} of about 25. Cells, kept on ice, were disrupted by 15 cycles of sonication (one cycle consisted of 15 s of sonication and 45 s of resting) using a Branson (Danbury, Conn.) Sonifier 250 . Cell extract (supernatant) was obtained

after removal of cell debris by centrifugation of the disrupted cells for 5 min at 9,200 x *g*. Peptidase activity in washed cells, cell extract, or supernatant (concentrated 50-fold by ultrafiltration with an Amicon filter (cutoff, 10 kDa; Amicon Corp., Lexington, Mass.)) was determined at 30°C with L-alanyl-*p*-nitroanilide, L-leucyl-*p*-nitroanilide, and L-lysyl-*p*-nitroanilide as described previously (11).

Transport assays in *L. monocytogenes*. (*i*) Transport of [^{14}C]ALA, prolyl-[^{14}C]alanine, and [^{14}C]alanine. Cells were grown in BHI, unless indicated otherwise, at 30°C with agitation (200 rpm) in a shaker-incubator (Gallenkamp, Griffin Europe, Breda, The Netherlands). Cells were harvested, washed twice, and resuspended (OD_{600}, 20) in 50 mM potassium phosphate buffer (pH 6.9)-5 mM $MgSO_4$ containing 50 μg of chloramphenicol per ml and stored on ice until use. Transport assays were conducted at 30°C in 0.1-ml incubation mixtures containing 0.01 ml cell of suspension and 0.5% (wt/vol) glucose in this buffer unless stated otherwise. After incubation, the uptake was stopped by the addition of 2 ml ice-cold buffer, and the cells were rapidly separated from the medium by filtration through 0.2-μm-pore-size cellulose nitrate filters (Schleicher & Schuell GmbH, Dassell, Germany) under vacuum and washed once more with 2 ml cold buffer. The radioactivity retained on the filters was measured subsequently in a liquid scintillation counter (model 1600TR; Packard Instruments Co., Downers Grove, Ill.).

***(ii)* Peptide transport determined with fluorescamine.** The fluorescamine procedure for monitoring peptide uptake based on methods described by Higgins and Gibson (15) and Perrett *et al.* (31) was modified for microtiter plates. Cells were harvested during logarithmic growth (OD_{600}, 0.6), washed twice, resuspended (OD_{600}, 20) in 50 mM potassium phosphate buffer (pH 6.9) containing 5 mM $MgSO_4$, and stored on ice until assayed. Transport assays were performed at 30°C in this buffer containing 0.5% (wt/vol) glucose. Cells (OD_{600}, 2) were preincubated for 10 min, and uptake was started by addition of the peptide. Samples were removed from the mixture after appropriate time intervals and immediately passed through 0.2-μm-pore-size cellulose nitrate filter (Schleicher & Schuell) to remove cells with a manifold filtration apparatus (Millipore Corp., Bedford, Mass.). Aliquots (40 μl) of the filtered medium were added to 300 μl 0.1 M sodium tetraborate buffer (pH 6.2). Freshly prepared fluorescamine solution in acetone (160 μl; 0.15 mg/ml) was added while the solution was being rapidly vortexed. Samples were transferred to the wells of a microtiter plate, and fluorescence was determined within 10 min at room temperature with a luminescence spectrometer (model LS50B; Perkin-Elmer Corp., Norwalk, Conn.) with excitation and emission wavelengths of 390 nm and 475 nm, respectively. Peptide concentrations were calculated from standard solutions of the individual peptide. The relation between the amount of any particular peptide and its fluorescence was shown to be linear in the concentration range used. Fluorescence of free amino acids was negligible (< 1%).

***(iii)* Peptide transport determined with high-performance liquid chromatography (HPLC).** Cells grown in BHI or DM were harvested at OD_{600} of 0.6, washed twice, and resuspended in 50 mM potassium phosphate buffer (pH 6.9)-5 mM $MgSO_4$ and stored on ice until use. Uptake of peptides was monitored by determining extracellular concentrations of residual peptide after removal of the cells (see above). Peptides and amino acids were analyzed after derivatization with dansyl chloride based on methods described by Tapuhi *et al.* (43) and Wiedmeier *et al.* (50). Dansylation of the samples was started by adding dansyl chloride, dissolved in acetonitrile, to a final concentration of 0.5 mg/ml. After 2 h of incubation at 37°C, the reaction was stopped by adding methylamine to 20 mM. The amount of dansylated amino acids and peptides was determined by reversed-phase HPLC on a C_{18} column (Spher 10ODS; 25 cm by 4.6 mm; Chrompack, Bergen op Zoom, The Netherlands) at a flow rate of 1 ml/min with a mobile phase of 55:45 (vol/vol) acetonitril-30 mM sodium phosphate

(pH 6.5). Peak areas were calibrated with standard solutions. The chromatographic apparatus (Spectra Physics, San Jose, Calif.) consisted of an SP8700 XR solvent delivery system, an SP8780 XR autosampler, and a Spectra system UV 2000 for detection at a wavelength of 280 nm. Output from the detector was recorded on an SP4270 integrator.

Fate of accumulated prolyl-[^{14}C]alanine. Cells grown at 30°C in DM were harvested at OD_{600} of 0.6, washed two times, and resuspended (OD_{600}, 20) in 50 mM potassium phosphate (pH 6.9) with 5 mM $MgSO_4$ and 50 μg of chloramphenicol per ml and stored on ice. After preincubation of cells (OD_{600}, 2.0) for 5 min, uptake was started by addition of prolyl-[^{14}C]alanine (final concentration 21 or 460 μM) in the absence or presence of 2 mM L-alanine. After 30 min, 600-μl samples were taken, and the cells were separated from the external medium by centrifugation (10 min, 8,000 x *g*). The supernatant was stored on ice, and cell pellets were washed twice with 50 mM potassium phosphate containing 5 mM $MgSO_4$ and 50 μg of chloramphenicol per ml. Radioactivity was extracted subsequently with 20 μl 5% (vol/vol) perchloric acid and 10 mM EDTA on ice for 45 min. The extracts were neutralized with an equal amount of 1 M KOH and $KHCO_3$. Aliquots of 12 μl of perchloric acid extracts and supernatant were chromatographed on silica gel (E. Merck AG, Darmstadt, Germany, or Whatmann, Maidstone, England) thin-layer plates with a butanol-acetic acid-acetone-H_2O (7:2:7:4) solvent system (6). Radioautographs were made with Kodak X-ray films.

Measurement of the membrane potential and intracellular ATP concentration. The transmembrane electrical potential ($\Delta\psi$) was determined with an electrode specific for the lipophylic cation tetraphenylphosphonium (final concentration, 4 μM), as described previously (38). Cells of *L. monocytogenes* were prepared for measurements as described above and incubated (OD_{600}, 1.9) at 30°C in 50 mM potassium phosphate (pH 6.9) in the presence of 0.5% (wt/vol) glucose, unless indicated otherwise. By adding the potassium proton exchanger nigericin (1 μM), the pH gradient (alkaline inside) was dissipated such that the PMF was composed of the $\Delta\psi$ only. The intracellular ATP concentration was determined as described previously (1). In experiments in which *N,N'*-dicyclohexylcarbodiimide (DCCD) and the phosphate analogs arsenate and vanadate were used, the potassium phosphate was replaced by 50 mM potassium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5).

Protein determination. Protein concentrations were determined by the method of Bradford (8) or Lowry *et al.* (21) with bovine serum albumin as a standard.

Chemicals. [^{14}C]ALA hydrochloride (1.8 TBq/mol) was obtained from DuPont. L-[U-^{14}C]alanine (5.9 TBq/mol) was purchased from the Radiochemical Centre, Amersham, Buckinghamshire, United Kingdom. Prolyl-[U^{14}C]alanine, ß-chloroalanine, alanyl-ß-chloroalanine, and alanyl-alanyl-ß-chloroalanine were a generous gift from the Department of Microbiology, Section Molecular Microbiology, University of Groningen, The Netherlands. All amino acids and peptides were obtained in the L configuration from Sigma Chemical Company, St. Louis, Mo. All other chemicals were reagent grade and obtained from commercial sources.

Results

Growth on essential amino acid-containing dipeptides. *L. monocytogenes* Scott A can grow rapidly in DM, while removal of the essential amino acids leucine (Fig. 1A) or methionine (Fig. 1B) results in an almost complete inhibition of growth. Upon addition of the dipeptides alanyl-leucine and leucyl-proline, growth in DM without leucine is stimulated, and both growth rate and final OD_{550} are comparable to those in DM (Fig. 1A). Growth in

DM without methionine is stimulated by prolyl-methionine and alanyl-methionine, but the latter dipeptide had a smaller effect (Fig. 1B). These results indicate that dipeptides can function as a source of essential amino acids. These experiments do not allow us to discriminate between (*i*) uptake of dipeptide followed by intracellular hydrolysis and (*ii*) extracellular dipeptide hydrolysis followed by uptake of free amino acids. To discriminate between these two possibilities, the effect of toxic amino acid and peptide analogs was investigated.

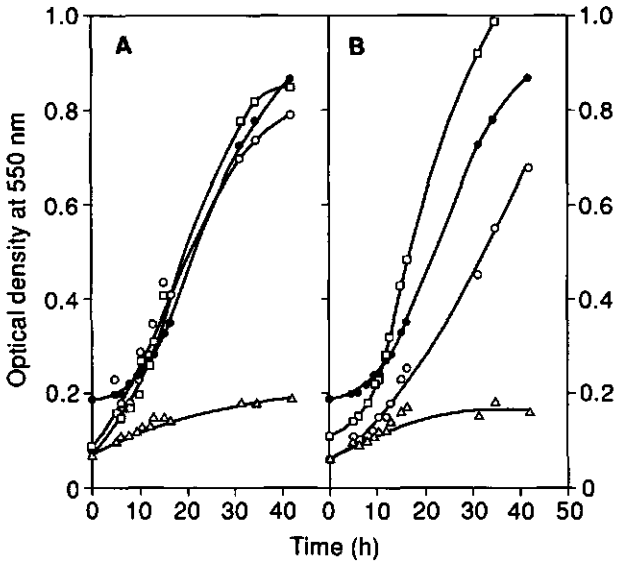

Figure 1. Leucine-containing (A) and methionine-containing (B) dipeptides as sources of essential amino acids for growth of *L. monocytogenes* Scott A. (A) *L. monocytogenes* was grown at 30°C in DM (●), in DM without the essential amino acid leucine (Δ), and in DM in the absence of leucine but with 0.1 mM alanyl-leucine (□) or 0.1 mM leucyl-proline (○). (B) *L. monocytogenes* was grown at 30°C in DM (●), DM without methionine (Δ), and DM without methionine but with 0.1 mM prolyl-methionine (□) or 0.1 mM alanyl-methionine (○). Growth was monitored spectrophotometrically at an OD_{550}.

Effects of chlorinated compounds on growth. ß-Chloroalanine irreversibly inhibits the enzyme alanine racemase (EC 5.1.1.1.) responsible for the formation of D-alanine in bacterial cells and thereby precludes the synthesis of a cell wall constituent necessary for bacterial growth (22). The racemization of L-alanine to D-alanine occurs in the cytoplasm (36). The toxicity of ß-chloroalanine, alanyl-ß-chloroalanine, and alanyl-alanyl-ß-chloroalanine in DM was tested on confluently plated *L. monocytogenes* Scott A cultures. Strikingly, the chlorinated amino acid ß-chloroalanine proved not to inhibit growth of *L. monocytogenes,* while large clear zones were observed in the presence of the peptide analogs alanyl-ß-chloroalanine and alanyl-alanyl-ß-chloroalanine, indicating that significant growth

inhibition occurred (data not shown).

Additional studies were performed in liquid media to assess the concentration dependence and the effect of nontoxic amino acid and peptide competitors on the inhibition of growth of *L. monocytogenes* by ß-chloroalanine and alanyl-ß-chloroalanine (Fig. 2). The addition of ß-chloroalanine (final concentration, 0.1 mM) resulted in a slight inhibition of growth (Fig. 2A). In the presence of 1 mM alanine, the inhibition by ß-chloroalanine was completely overcome, and growth was comparable to that in DM. The finding that ß-chloroalanine did not exhibit toxicity towards *L. monocytogenes* indicates that it cannot enter the cell under these conditions. This might be due to an excess of other (nontoxic) amino acids in DM that compete for uptake or to a low affinity of the alanine transporter for ß-chloroalanine. Indeed, uptake of [^{14}C]alanine by glucose-energized cells in potassium phosphate (pH 6.9) was significantly reduced in the presence of excess amino acids present in DM (see Materials and Methods; data not shown).

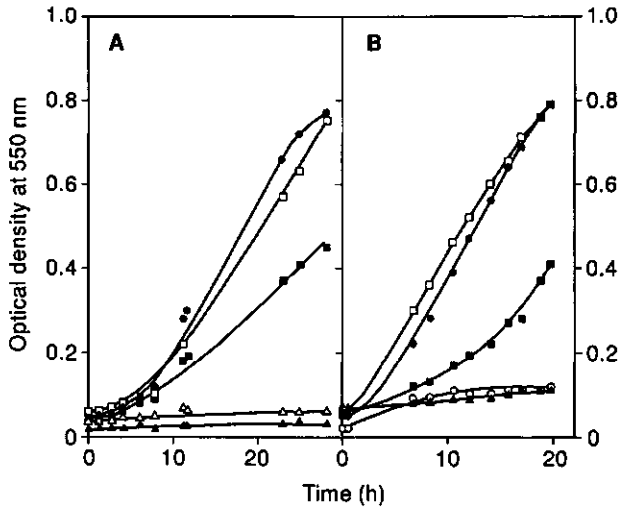

Figure 2. Effect of ß-chloroalanine and alanyl-ß-chloroalanine on growth of *L. monocytogenes* Scott A. *L. monocytogenes* was grown at 30°C in DM, and growth was monitored spectrophotometrically at an OD_{550}. (A) Growth was determined in DM (●), in DM containing 0.1 mM ß-chloroalanine in the absence (■) and presence (□) of 1.0 mM alanine, and in DM containing 0.1 mM alanyl-ß-chloroalanine in the absence (▲) and presence (Δ) of 1 mM alanine. (B) Growth was determined in DM (●) and in DM containing 0.02 mM alanyl-ß-chloroalanine in the absence (▲) and presence of 0.2 mM (○), 2.0 mM (■), and 10 mM (□) alanyl-alanine.

When the toxic compound was supplied in a peptide configuration, *i.e.*, in the form of the peptide analog alanyl-ß-chloroalanine, it appeared to act much more efficiently and inhibited growth of *L. monocytogenes* completely at a concentration of 0.01 mM (data not shown). In the presence of 0.1 mM alanyl-ß-chloroalanine, growth inhibition could not be

relieved upon addition of excess alanine (1 mM) (Fig. 2A). The addition of the dipeptide alanyl-alanine did not stimulate growth at low concentrations (final concentration, 0.2 mM). However, when the toxic dipeptide containing DM was supplemented with 2 mM of alanyl-alanine (hundredfold excess), growth suppression by alanyl-ß-chloroalanine was diminished, and at a concentration of 10 mM alanyl-alanine, growth was comparable to that in DM (Fig. 2B). The addition of the dipeptide alanyl-methionine or leucyl-proline to a final concentration of 2 mM in DM containing 0.02 mM alanyl-ß-chloroalanine did not result in reversal of growth inhibition by the toxic dipeptide. Only at very high concentrations (16 mM) was alanyl-methionine able to diminish the toxic effect of alanyl-ß-chloroalanine (data not shown). The efficient inhibition of growth of *L. monocytogenes* by alanyl-ß-chloroalanine combined with the observation that the presence of excess nontoxic dipeptide relieves growth inhibition, whereas alanine does not, indicate that the toxic amino acid moiety (ß-chloroalanine) enters the cell as a dipeptide.

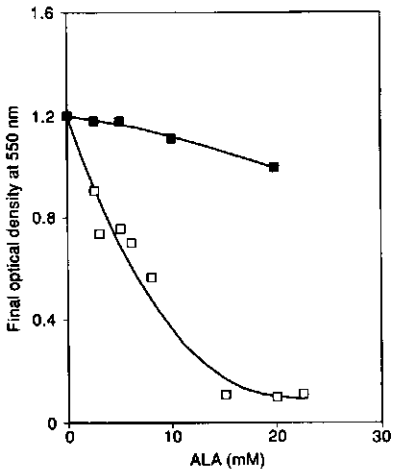

Figure 3. Effect of ALA on growth of *L. monocytogenes* in DM (■) and in DM without methionine but with alanyl-methionine (□). The final OD_{550} was determined as a measure of growth in the presence of increasing concentrations of ALA in DM (■) and in DM without methionine but in the presence of the dipeptide alanyl-methionine (0.1 mM) (□).

Effect of ALA on growth of *L. monocytogenes* in DM and in DM without methionine but with L-alanyl-L-methionine. Recently, it was reported that the dipeptide analog ALA is a substrate for the dipeptide permease (Dpp) of the gram-negative bacteria *Salmonella typhimurium* (10) and *Escherichia coli* (49). ALA closely resembles the dipeptide glycyl-glycine but does not contain a peptide bond and therefore is not hydrolyzed by peptidases (10). The effect of increasing concentrations of ALA on growth of *L. monocytogenes* was studied in DM and in DM in which the dipeptide alanyl-methionine (final concentration, 0.1

mM) was present as the source of methionine (Fig. 3). Even at very high concentrations, ALA showed only a minor effect on growth of *L. monocytogenes* in DM, while growth in DM containing alanyl-methionine was significantly inhibited (Fig. 3). Evidently, the presence of large amounts of ALA prevent alanyl-methionine uptake by the cells, and consequently, growth is inhibited since the cells are not supplied with the essential amino acid methionine. This suggests that ALA competes with alanyl-methionine for uptake by a dipeptide transport system.

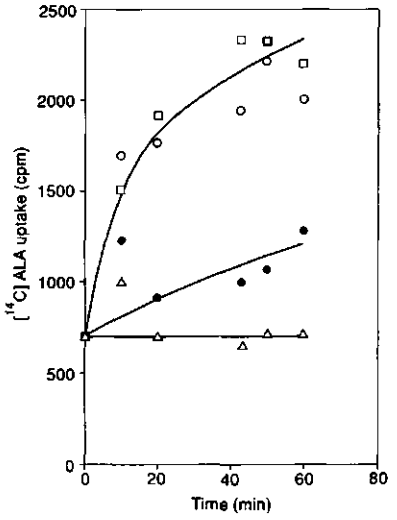

Figure 4. Uptake of [^{14}C]ALA in *L. monocytogenes*. Cells were grown in DM in which alanyl-methionine served as the source of methionine. Uptake of [^{14}C]ALA (final concentration, 21 μM) was started after preincubation for 5 min. Transport assays were performed at 30°C with cells incubated in 50 mM potassium phosphate (pH 6.9) in the absence of glucose (□) in the presence of 0.5% (wt/vol) glucose (○), and in the presence of 0.5% (wt/vol) glucose with 3 mM ALA (●) or with valinomycin and nigericin (final concentrations, 1 μM) (Δ).

Transport of [^{14}C]ALA in *L. monocytogenes*. Uptake of [^{14}C]ALA was investigated in cells of *L. monocytogenes*. Transport of [^{14}C]ALA (final concentration, 21 μM) was detected in cells incubated in 50 mM potassium phosphate (pH 6.9) both in the absence and presence of glucose (Fig. 4). The accumulation of [^{14}C]ALA was significantly reduced in the presence of a 140-fold excess (3 mM) of unlabeled ALA because of competition for the transport system. Uptake of [^{14}C]ALA was also significantly inhibited in the presence of the potassium ionophore valinomycin (final concentration, 1 μM) and the potassium proton exchanger nigericin (final concentration, 1 μM) (Fig. 4). Under these conditions, the PMF is dissipated (data not shown) and the inhibition of [^{14}C]ALA transport indicates that uptake of ALA via the proposed dipeptide transport system is energy dependent.

Characterization of a di- and tripeptide transport system in *L. monocytogenes*. (*i*) Transport of prolyl-[^{14}C]alanine. Dipeptide transport in BHI-grown cells of *L. monocytogenes* was assayed with radioactively labeled prolyl-[^{14}C]alanine. A significant uptake of prolyl-[^{14}C]alanine (final concentration, 21 μM) was observed in glucose-energized cells. Transport was strongly inhibited in the presence of excess unlabeled prolyl-alanine (final concentration, 1 mM), whereas transport was stimulated in the presence of alanine (1 mM) (Fig. 5). These results indicate that the dipeptide rather than [^{14}C]alanine is transported; [^{14}C]alanine could have been formed from extracellular peptidase activity (see also below). Prolyl-[^{14}C]alanine was also taken up at a high rate in cells grown in DM, indicating that the transport system is constitutively expressed rather than induced during growth in the peptide-containing BHI (data not shown). Experiments in which metabolism of accumulated prolyl-[^{14}C]alanine was investigated showed that the dipeptide was hydrolyzed intracellularly, although faint spots of the peptide could also be detected in autoradiographs after thin-layer chromatography of CEs (data not shown). The apparent increase in accumulation of prolyl-[^{14}C]alanine in the presence of excess alanine can be explained by an inhibition of efflux and/or metabolism of [^{14}C]alanine, resulting in efflux of ^{14}C-labeled metabolites, due to high intracellular concentrations of unlabeled alanine previously accumulated via an alanine transport system (see above). Rapid uptake of prolyl-alanine was also inferred from HPLC analysis of supernatants of glucose-energized cell suspensions, and the initial rate of uptake of prolyl-alanine (final concentration, 0.3 mM) was 12 nmol/min/mg of protein. After prolonged incubation (at 20 min) 0.02 mM proline (approximately 15 % of the total amount of prolyl-alanine taken up by the cells), but not alanine, was detected in the medium. Proline was most likely lost from the cells after intracellular hydrolysis of accumulated dipeptide.

***(ii)* Kinetic parameters of prolyl-alanine transport.** Kinetic analysis of prolyl-[^{14}C]alanine transport in glucose-energized cells of *L. monocytogenes* revealed the presence of a single transport system with a Michaelis-Menten constant of about 520 μM and a corresponding V_{max} of 14.2 nmol/min/mg of protein (data not shown).

***(iii)* Energetics of prolyl-alanine transport.** In glucose-energized cells of *L. monocytogenes* at pH 6.9, a $\Delta\psi$ (negative inside) of -80 mV and a pH gradient (ΔpH) of -55 mV (intracellular pH, 7.8) were determined, resulting in a PMF of -135 mV (negative and alkaline inside; data not shown). Under these conditions, a rapid uptake of prolyl-[^{14}C]alanine was observed (Fig. 6). Transport is not significantly affected by the presence of nigericin (1 μM), which results in a dissipation of the pH gradient that is compensated for by an increase in the $\Delta\psi$ from -80 to -129 mV (PMF = $\Delta\psi$ = -129 mV). Valinomycin (1 μM) dissipates the $\Delta\psi$ while the pH gradient increases from -55 mV to -75 mV, resulting in a somewhat lower PMF than that of the control (PMF = ΔpH = -75 mV) and a reduction in dipeptide transport (approximately 30% of the activity of the control). The addition of both valinomycin (1 μM) and nigericin (1 μM) results in a dissipation of the PMF and a total inhibition of prolyl-[^{14}C]alanine transport (Fig. 6). Since the intracellular ATP concentrations were approximately the same under the various conditions (about 8 mM; data not shown), these results suggest that dipeptide transport is coupled to the PMF.

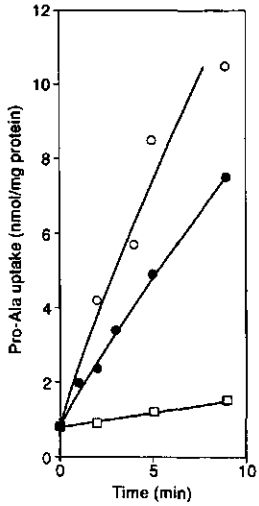

Figure 5. Transport of prolyl-[^{14}C]alanine (Pro-Ala) in *L. monocytogenes* in the absence or presence of 1 mM unlabeled substrate. Uptake of prolyl-[^{14}C]alanine (final concentration, 21 μM) was performed in 50 mM potassium phosphate (pH 6.9) containing 0.5% (wt/vol) glucose, 5 mM $MgSO_4$ and 50 μg of chloramphenicol per ml. Uptake was started after preincubation for 5 min by the addition of prolyl-[^{14}C]alanine in the absence (●) and presence (○) of 1 mM alanine or in the presence of 1 mM prolyl-alanine (□).

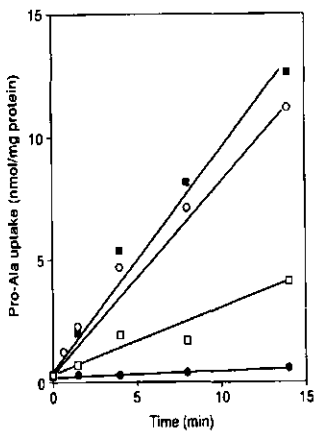

Figure 6. Energetics of prolyl-[^{14}C]alanine transport in *L. monocytogenes*. Uptake of prolyl-[^{14}C]alanine was performed in glucose-energized cells as described in the legend to Fig. 5. Symbols: (○), no addition; (■), 1 μM nigericin added; (□), 1 μM valinomycin added; (●), 1 μM nigericin and 1 μM valinomycin added.

The mode of energy coupling to the transport of prolyl-alanine was studied in greater detail by analysis of the effects of the phosphate analogs arsenate and vanadate and the H^+-ATPase inhibitor DCCD on bioenergetic parameters and dipeptide transport in *L. monocytogenes*. The addition of DCCD resulted in a complete dissipation of the PMF, while the intracellular ATP concentration was increased (145 % of control). Apparently, the PMF is generated mainly upon H^+ extrusion by the membrane-bound ATPase. Prolyl-alanine transport is almost completely inhibited under these conditions (Table 1). Dipeptide transport was not affected significantly by arsenate or vanadate, and again no correlation with the intracellular ATP concentration was found. Slight inhibition of transport in the presence of vanadate (78 % of the activity of the control) is most likely caused by the reduction in the PMF. Combining these results, we can conclude that prolyl-alanine transport in *L. monocytogenes* is coupled to the PMF.

Table 1. Effect of DCCD, arsenate and vanadate on bioenergetic parameters and prolyl-[^{14}C]alanine uptake in *Listeria monocytogenes*[a].

ATPase inhibitor or phosphate analog added[b]	PMF (mV)	ATP_{in}[c] (mM)	Prolyl-[^{14}C]alanine uptake (nmol/min/mg of protein)
none	-128 (100)[d]	7.1 (100)	0.31 (100)
DCCD (0.1 mM)	0 (0)	10.2 (145)	0.02 (6)
arsenate (0.2 mM)	-128 (100)	5.4 (78)	0.29 (96)
vanadate (0.2 mM)	-118 (90)	6.5 (92)	0.24 (78)

[a] The initial rate of uptake of prolyl-[^{14}C]alanine (final concentration, 20 μM) was determined over the first 10 min in BHI-grown cells incubated at 30°C in 50 mM potassium HEPES (pH 7.5) containing 0.5% (wt/vol) glucose, 5 mM $MgSO_4$, and 50 μg of chloramphenicol per ml. The potassium proton exchanger nigericin was added (final concentration, 1 μM) to dissipate the pH gradient (ΔpH) so that the PMF was composed of the membrane potential only (PMF = $\Delta\psi$). Uptake was started after preincubation for 10 min by addition of prolyl-[^{14}C]alanine. The $\Delta\psi$ was determined in parallel experiments, and samples were taken for determination of the intracellular ATP concentrations.

[b] DCCD, arsenate, or vanadate was added to the indicated final concentrations, after preincubation for 5 min. After incubation for another 5 min, uptake was started by the addition of prolyl-[^{14}C]alanine.

[c] ATP_{in}, intracellular ATP concentration.

[d] The values for PMF, intracellular ATP concentration (ATP_{in}), and initial rate of uptake of prolyl-[^{14}C]alanine in the control experiment (no addition) were set at 100%. Relative percentages in the presence of inhibitors are given in parentheses.

***(iv)* Substrate specificity of the prolyl-alanine transport system.** Uptake of prolyl-[^{14}C]alanine (final concentration, 420 μM) by *L. monocytogenes* was determined in the presence of excess unlabeled peptide. In addition to prolyl-alanine, transport was significantly

inhibited by the dipeptides leucyl-proline and alanyl-alanine, while prolyl-glycine and alanyl-methionine were less inhibitory and alanyl-glutamate was the least effective. Uptake of radioactively labeled dipeptide was also decreased in the presence of excess tripeptide; alanyl-alanyl-alanine was the most effective, and the tetrapeptide alanyl-leucyl-alanyl-leucine was not an efficient inhibitor (Table 2). From these results, we can conclude that di- and tripeptides compete for the same transport system, with leucyl-proline, alanyl-alanine and alanyl-alanyl-alanine as the high-affinity substrates. Analysis of peptide uptake (final concentration, 0.5 mM) with fluorescamine revealed that the latter peptides were transported at high rates in glucose-energized cells of *L. monocytogenes* (initial rates of uptake, approximately 80 to 100 nmol/min/mg of protein), while the rate of uptake of the low-affinity substrate alanyl-glutamate was much lower (approximately 12 nmol/min/mg of protein; data not shown).

Table. 2. Uptake of prolyl-[^{14}C]alanine by *L. monocytogenes* in the presence of excess unlabeled peptide.

Peptide	Uptake of prolyl-[^{14}C]alanine (% activity)[a]	
	10 x unlabeled peptide	100 x unlabeled peptide
Prolyl-alanine	16	5
Prolyl-glycine	44	24
Leucyl-proline	8	-[b]
Alanyl-alanine	2	0
Alanyl-methionine	55	-
Alanyl-glutamate	86	-
Alanyl-alanyl-alanine	16	3
Alanyl-leucyl-alanine	44	-
Prolyl-glycyl-glycine	63	60
Alanyl-leucyl-alanyl-leucine	81	-

[a] Uptake of prolyl-[^{14}C]alanine (final concentration, 420 μM) was determined in the presence of 10- and 100-fold excess of simultaneously added unlabeled peptide. The initial rate of uptake was determined over the first 10 min in BHI-grown cells after preincubation for 10 min in 50 mM potassium phosphate (pH 7.0) containing 0.5% (wt/vol) glucose, 5 mM $MgSO_4$, and 50 μg of chloramphenicol per ml. One hundred percent activity, *i.e.*, the initial rate of prolyl-[^{14}C]alanine uptake (final concentration, 420 μM) at 30°C was 2.1 nmol/min/mg of protein.
[b] -, not determined

Peptidase activities in CEs of *L. monocytogenes*. Peptidase activities towards various synthetic substrates could be detected in CEs obtained from *L. monocytogenes* cells grown

in BHI or in DM (Table 3). The highest peptidase activity in CEs from BHI-grown cells was found with lysyl-*p*-nitroanilide, whereas CEs from DM-grown cells had the highest activity for alanyl-*p*-nitroanilide. This observation indicates that the intracellular peptidase activities are influenced by the composition of the growth medium. Intact cells exhibited a higher activity (approximately fivefold) than that found in an equivalent amount of disrupted cells (data not shown). Loss of peptidase activity due to the fractionation step or a more optimal environment for enzyme activity inside the cell may account for the observed differences in peptidase activity between whole cells and CEs. Aminopeptidase activities could not be detected in 50-fold-concentrated cell-free supernatant from BHI- or DM-grown cultures, which indicates that extracellular dipeptide hydrolysis does not occur.

Table 3. Specific peptidase activities in CEs from cells of *Listeria monocytogenes* grown in BHI or in DM

Substrate	Specific activity (pmol/min/mg of protein) in CEs grown in[a]:	
	BHI	DM
L-Alanyl-*p*-nitroanilide (2 mM)	200	800
L-Leucyl-*p*-nitroanilide (2 mM)	300	200
L-Lysyl-*p*-nitroanilide (2 mM)	400	400

[a] Peptidase activities were determined at 30°C in 50 mM potassium phosphate (pH 6.9). The reactions proceeded linearly with time under the conditions of the assay. CEs were prepared from cells grown in BHI or in DM at 30°C and harvested during exponential growth at an OD_{600} of 0.6.

Discussion

In this report, we demonstrate that dipeptides are nutritionally valuable in providing *L. monocytogenes* with essential amino acids. Several observations suggest that these dipeptides are translocated via a specific di- and tripeptide transport system into the cell prior to hydrolysis. (*i*) The toxic effect of the dipeptide analog alanyl-ß-chloroalanine on growth of *L. monocytogenes* was completely prevented by an excess of dipeptides, whereas excess alanine was without effect. This indicates that the toxic dipeptide is taken up by the cell via the same system that transports nontoxic dipeptides. Alanyl-ß-chloroalanine exerts its toxicity most likely after intracellular hydrolysis, thereby liberating the toxic amino acid ß-chloroalanine. The finding that ß-chloroalanine did not cause growth inhibition of *L. monocytogenes* in DM is most likely due to the presence of excess nontoxic amino acids in DM. This excludes the involvement of an extracellular peptidase (*e.g.*, aminopeptidase) in combination with an amino acid transport system but points to alanyl-ß-chloroalanine uptake

followed by intracellular hydrolysis of the peptide. (*ii*) The growth experiments with the porphyrin precursor ALA, whose structure is closely related to that of glycyl-glycine (10), revealed that ALA competes with the dipeptide alanyl-methionine for uptake. (*iii*) The uptake of radioactively labeled prolyl-[^{14}C]alanine is specifically inhibited in the presence of excess unlabeled di- and tripeptides. (*iv*) Aminopeptidase activities could be detected only in CEs and with whole cells of *L. monocytogenes*, whereas activity was not detected in 50-fold-concentrated supernatant from BHI- and DM-grown cultures, which indicates that extracellular peptide hydrolysis does not occur.

High rates of dipeptide uptake were observed in cells of *L. monocytogenes* grown in DM (data not shown). This indicates that the di- and tripeptide transport system is probably constitutive, which is in line with other observations on dipeptide transport in various gram-negative and gram-positive bacteria (29, 40). *Bacillus subtilis* is an exception to this rule, since the dipeptide and oligopeptide transport systems are expressed only under conditions that cause the cells to enter stationary phase and initiate sporulation (25, 30, 39). *L. monocytogenes* may have evolved special adaptations to grow on peptides since it cannot utilize proteins as a source of amino acids. These adaptations allow the organism to grow in foods that are poor in free amino acids but rich in peptides as a consequence of proteolytic activity of other microorganisms present in such foods. Furthermore, *L. monocytogenes* does not have to spend energy on the synthesis of a protease(s), which might offer a competitive advantage over microorganisms that synthesize these proteins. For example, protease-negative (Prt$^-$) variants were shown to outperform wild-type strains of *Lactococcus* strains (Prt$^+$) at pH values higher than 6.0 in milk. Under these conditions, essential nutrients are supplied by the remaining 5% of the Prt$^+$ variant (16). The zinc-dependent metalloprotease (Mpl), which is one of the virulence factors in *L. monocytogenes*, was recently shown to be involved in processing phosphatidylcholine-specific phospholipase C (PlcB) from the precursor to its active form (32). Whether this protease is involved in the supply of *L. monocytogenes* peptide nutrients during intracytoplasmic growth in mammalian cells remains to be elucidated (see also below).

Detailed characterization of the energetics of the di- and tripeptide uptake system in *L. monocytogenes* revealed that transport is coupled to the PMF (Fig. 6 and Table 2). This peptide transport system of *L. monocytogenes* resembles, therefore, the PMF-dependent di- and tripeptide transport system in *Lactococcus lactis* (40). The gene encoding the lactococcal di- and tripeptide transport system (*dtpT*) was recently cloned and sequenced. The translated sequence corresponds to that of a protein of 463 amino acid residues, and hydropathy profiling indicated that the protein could form 12 membrane-spanning segments typical for secondary transport systems (14). All other bacterial peptide transport systems described to date, including the oligopeptide transport system in *L. lactis* (18, 46), are members of a larger family, the ABC transporter or traffic ATPase superfamily. Transport of peptides via these multicomponent, binding protein-dependent permeases is driven by ATP (2, 3, 41).

The di- and tripeptide transport system in *L. monocytogenes* evidently exhibits broad specificity with regard to the nature of the amino acids that constitute the peptide since it allows transport of the nonpeptide substrate ALA and the di- and tripeptide analogues alanyl-

ß-chloroalanine and alanyl-alanyl-ß-chloroalanine, respectively, in addition to different di- and tripeptides. The DtpT system of *L. lactis* has also a broad substrate specificity, whereas proline-containing dipeptides especially are transported at high rates (40). The uptake rates of zwitterionic dipeptides in cells of *L. monocytogenes* are in the same order of magnitude as those described for *L. lactis* ML3 (14, 18, 40), whereas the specific aminopeptidase activity reported for lactococci is 50 to 100-fold higher (6, 42). Although the role of other peptidases in *L. monocytogenes* that are not active towards the synthetic *p*-nitroanilides used cannot be ruled out completely, it is conceivable that the aminopeptidase activity in lactococci is relatively high because this group of bacteria exist by virtue of its proteolytic power (6, 44). The uptake of dipeptides is the rate-limiting step in dipeptide utilization in lactococci (40, 47). Our results on peptidase activity suggest that in *L. monocytogenes* cells, hydrolysis of the peptide bond might be rate limiting. This proposal is strengthened by the findings of (*i*) small amounts of prolyl-[^{14}C]alanine in cells of *L. monocytogenes* after uptake of the radioactively labeled dipeptide (data not shown) and (*ii*) the presence of peptides in cells of *L. monocytogenes* after growth in peptone and in DM with proline containing di- and tripeptides (4) (see below).

L. monocytogenes can grow under anaerobic and aerobic conditions. Analysis of the respiratory activity in aerobically grown cells suggested the presence of a membrane-associated respiratory chain (27). However, cytochromes were not detected by reduced *minus* oxidized difference spectra at room temperature of whole cells and lysed protoplasts. Earlier reports also noted either the absence or presence of very low amounts of cytochromes (37, 45). The possible conservation of energy in the form of a PMF generated upon proton extrusion by electron transfer chain activity is unknown (27). However, in our experiments with glucose-energized cells of *L. monocytogenes* grown under aerobic conditions in BHI, the PMF was abolished completely upon addition of the H^+-ATPase inhibitor DCCD indicating that electron transfer chain activity does not contribute significantly to PMF generation (Table 1). Recently, growth of *E. coli* and *S. typhimurium* mutants defective in synthesis of the heme precursor ALA (*hemA* or *hemL*) was shown to be dependent on the exogenous supply of ALA, which was taken up via the dipeptide transport systems in these organisms (10, 49). The apparent broad substrate specificity of the di- and tripeptide transport system apparently also allows *L. monocytogenes* to take up ALA via this system (Fig. 3 and 4). The possible role of this process in cytochrome synthesis in *L. monocytogenes* requires further investigation.

The finding that growth of *L. monocytogenes* is completely inhibited in the presence of the dipeptide analog alanyl-ß-chloroalanine and the tripeptide analog L-alanyl-L-alanyl-ß-chloro-L-alanine offers new perspectives for the search for, and development of, peptide carrier prodrugs known as smugglins (28) that are active against this pathogen. There is an extensive literature concerning the use of peptide transport systems for uptake of antimicrobial agents (29). More detailed knowledge about substrate specificity and corresponding uptake activities of the di- and tripeptide transport system and possible other peptide transport systems in *L. monocytogenes* might allow the development of novel (single- or double-warhead) smugglins.

In conclusion, *L. monocytogenes* is equipped with a di- and tripeptide transport system for the translocation of small peptides that can provide the organism with essential amino acids. This allows *L. monocytogenes* to grow in foods that have amino acids in the form of peptides available as a result of the liberation from proteins by proteases of other microorganisms present in such foods. This finding might have other important implications since peptides can also serve other functions in bacteria in addition to being the nutrient source. Examples involve recycling of cell wall peptides (13), chemotaxis (17, 23) osmoregulation (4), and virulence (24, 29).

Recently, Amezaga *et al.* (4) showed that specific glycine and proline-containing di- and tripeptides stimulated growth of *L. monocytogenes* at high osmolarity. Under these conditions *L. monocytogenes* appeared to accumulate high levels of these peptides such that they contribute to osmoregulation. Prolyl-hydroxyproline and prolyl-glycyl-glycine were very effective and could indeed substitute for the osmoprotectant betaine (4). The di- and tripeptide uptake system in *L. monocytogenes* displays affinity for various proline-containing peptides (Table 2), which suggests that it might play an important role in osmoregulation of *L. monocytogenes* during growth at high osmolarity.

Furthermore, the described di- and tripeptide transport system may be important for intracytoplasmic growth of *L. monocytogenes* in mammalian cells. Microbial acquisition of nutrients is a central feature of host-parasite relationships, and bacterial pathogenicity is in part dependent on the availability and acquisition of nutrients. Investigation of the intracytoplasmic growth and virulence of *L. monocytogenes* auxotrophic mutants revealed that this food pathogen may utilize intracellular peptides as a source of amino acids (24).

For many gram-negative and gram-positive bacteria, separate transport systems for di- and tripeptides and oligopeptides have been described (29). The presence of other peptide transport systems in *L. monocytogenes* and their possible roles in growth under various environmental conditions are currently being investigated.

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3

Involvement of an ATP-dependent transport system in the utilization of oligopeptides by *Listeria monocytogenes* Scott A

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Abstract

For effective utilization of peptides, *Listeria monocytogenes*, possesses two different peptide transport systems. The first one is the earlier-described proton motive force (PMF) driven di-tripeptide transport system (A. Verheul, A. Hagting, M.-R. Amezaga, I. R. Booth, F. M. Rombouts, and T. Abee, Appl. Environ. Microbiol. 61:226-233, 1995). The present results reveal that *L. monocytogenes* possesses an oligopeptide transport system, requiring ATP rather than the PMF as the driving force for translocation. Growth experiments in a defined medium containing peptides of varying length suggested that the oligopeptide permease transports peptides of up to eight amino acid residues. Peptidase activities towards several oligopeptides were demonstrated in cell extract from *L. monocytogenes*, which indicates that upon internalization, the oligopeptides are hydrolyzed to serve as sources of amino acids for growth. The peptide transporters of the non-proteolytic *L. monocytogenes* might play an important role in foods that harbor endigenous proteinases and/or proteolytic microorganisms, since *Pseudomonas fragi* as well as *Bacillus cereus* were found to enhance the growth of *L. monocytogenes* to a large extent in a medium with the milk protein casein present as the sole source of nitrogen. In addition, growth stimulation was elicited in this medium when casein was hydrolyzed using purified protease from *Bacillus licheniformis*. The possible contribution of the oligopeptide transport system in the establishment of high numbers of *L. monocytogenes* in fermented milk products is discussed.

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exponential growth. Subsequently, cells were washed with 50 mM potassium phosphate (pH 6 or 6.9, as indicated) with 5 mM $MgSO_4$ and concentrated to an OD_{620} of approximately 20 and stored on ice until use. For transport assays, cells (OD_{620}, 2) were pre-incubated at 30°C for 5 min in the presence of 20 mM glucose, after which 0.3 mM peptide was added. Transport was monitored by determining extracellular concentrations of residual peptide after removal of the cells by centrifugation (75,000 x g for 30 s at 4°C) using a Biofuge Fresco eppendorfcentrifuge (Heraeus Instruments, Ostenrode, Germany) at various time intervals. Peptides and amino acids were dansylated and analysed by reversed-phase HPLC as described (50). In experiments in which the phosphate analog vanadate was used, the potassium phosphate was replaced by 50 mM potassium-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, pH 7.5).

Protein determination. Protein concentrations were determined by the method of Lowry *et al.* (28).

Chemicals. Peptides, ß-casein and Na-caseinate, containing α, ß and κ casein, were obtained from Sigma Chemical Co., St. Louis, Mo. or Bachem Feinchemikalien AG, Bubendorf, Switzerland. Chromogenic substrates were purchased from Bachem or from Chromogenix AB, Mölndal, Sweden. All other chemicals were reagent grade and obtained from commercial sources.

Results

Growth on valine-containing peptides. Growth of *L. monocytogenes* Scott A on specific peptides was measured using a defined minimal medium (DM) containing all essential amino acids except for valine, which was supplied in the form of a peptide. Several peptides containing between two and ten amino acid residues, with valine at different positions in the peptide chain, were used. *L. monocytogenes* failed to grow in DM lacking valine (Fig. 1A), while addition of certain valine-containing di-, tri-, tetra-, penta-, hexa-, hepta- and octapeptides to DM without valine resulted in restoration of growth (Fig 1B to 1H). The valine-containing nona- and decapeptides used in our study were ineffective in stimulating the growth of *L. monocytogenes* in DM without valine (Fig. 1I and 1J). These results indicate that oligopeptides can function as a source of essential amino acids for *L. monocytogenes* and suggest a size restriction for peptide utilization of eight amino acids.

Peptidase activity in CE of *L. monocytogenes*. If oligopeptide hydrolyzing enzymes are present externally, amino acids and di- and tripeptides will be formed which can subsequently be taken up by the corresponding transport systems (50), whereas intracellular accumulation of oligopeptides implicates the presence of a functional oligopeptide transport system. Incubation of CE obtained from DM-grown *L. monocytogenes* with hexa-alanine ($(Ala)_6$) resulted in the appearance of alanine in the assay mixture (Fig. 2A). This conversion of $(Ala)_6$ into alanine corresponds with a peptidase activity of 2 nmol min^{-1} mg of $protein^{-1}$ (not shown). Hydrolysis of $(Ala)_6$ could not be detected in 200-fold-concentrated supernatant obtained from *L. monocytogenes* grown in DM (Fig. 2B). Similarly, peptidase activity towards valine-containing peptides (*i.e.* Val-Gly (n=2), Val-Gly-Asp-Glu (n=4), Val-Leu-Ser-Glu-Gly (n=5), Arg-Val-Tyr-Ile-His-Pro-Phe (n=7), Pro-His-Pro-Phe-His-Leu-Phe-Val-Tyr (n=9), and Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu (n=10)) could be detected only

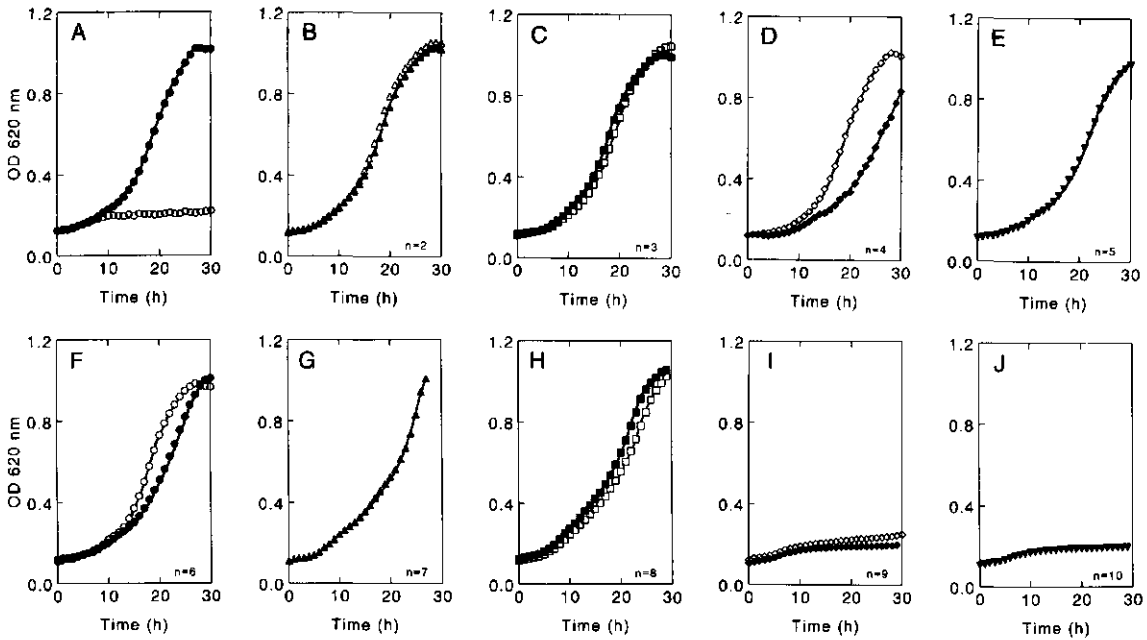

Figure 1. Valine-containing peptides as sources of essential amino acids for growth of *L. monocytogenes*. *L. monocytogenes* was cultured at 30°C and growth was monitored spectrophotometrically at an OD_{620}. (A) Growth in DM (●) and in DM without valine (○). (B) Growth in DM without valine with dipeptides: Val-Gly (▲) or Ala-Val (Δ). (C) Growth in DM without valine with tripeptides: Val-Pro-Leu (■) or Ala-Val-Leu (□). (D) Growth in DM without valine with tetrapeptides: Val-Gly-Asp-Glu (♦) or Val-Ala-Ala-Phe (◇). (E) Growth in DM without valine with the pentapeptide Val-Leu-Ser-Glu-Gly (▼). (F) Growth in DM without valine with hexapeptides: Val-Gly-Gly-Ser-Glu-Ile (●) or Gly-Ala-Val-Ser-Thr-Ala (○). (G) Growth in DM without valine with the heptapeptide: Arg-Val-Tyr-Ile-His-Pro-Phe (▲). (H) Growth in DM without valine with octapeptides: Val-His-Leu-Thr-Pro-Val-Glu-Lys (■) or Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (□). (I) Growth in DM without valine with nonapeptides: Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu (♦) or Pro-His-Pro-Phe-His-Leu-Phe-Val-Tyr (◇). (J) Growth in DM without valine with the decapeptide Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu (▼). n; number of amino acid residues in peptides.

in CE (several breakdown products were found including individual amino acids), and not in concentrated supernatant of *L. monocytogenes* (data not shown). The data indicate that the hydrolysis of oligopeptides occurs in the cytoplasm.

Peptidase activity in CE and 200-fold-concentrated supernatant of *L. monocytogenes* was also assayed by use of chromogenic substrates. A peptidase activity of 2 nmol min^{-1} mg of $protein^{-1}$ was found in CE with succinyl-alanyl-alanyl-prolyl-phenylalanyl-*p*-nitroanilide, whereas no release of nitroanilide could be detected upon incubation of CE with the other substrates (*i.e.* alanyl-prolyl-*p*-nitroanilide, glycyl-prolyl-*p*-nitroanilide, acyl-alanyl-alanyl-alanyl-*p*-nitroanilide, and isoleucyl-prolyl-arginyl-*p*-nitroanilide). None of the chromogenic substrates tested were hydrolyzed upon incubation with concentrated supernatant.

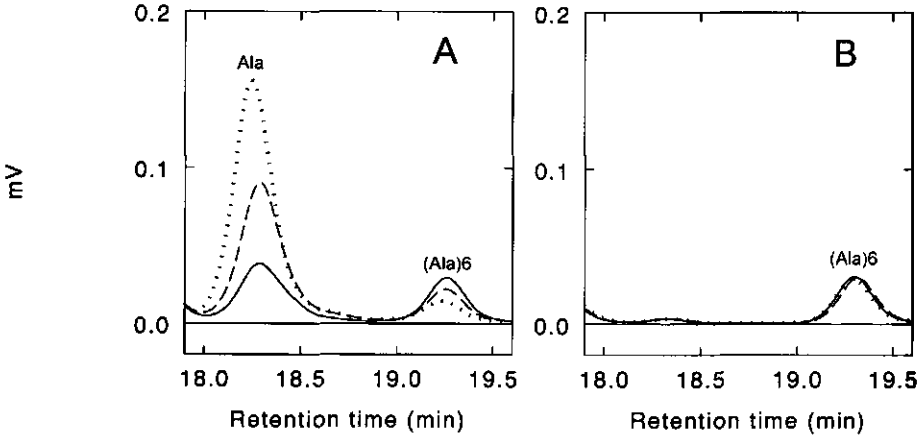

Figure 2. HPLC analysis of hexa-alanine ($(Ala)_6$) incubation with cell extract (CE) (A) and 200-fold-concentrated supernatant (B). CE (1.2 mg of protein ml^{-1}) and 200-fold concentrated supernatant (0.4 mg of protein ml^{-1}) was incubated with 0.5 mM $(Ala)_6$ in 50 mM potassium phosphate (pH 6.9) containing 5 mM $MgSO_4$ and samples were taken, and analyzed after derivatization with dansyl chloride by reversed-phase HPLC. (—), t = 0; (– –), t = 40 min; (‚‚‚), t = 90 min. CE and concentrated supernatant was prepared from cells grown in DM at 30°C and harvested during expontial growth.

Oligopeptide transport in *L. monocytogenes*. Uptake of the pentapeptide Val-Leu-Ser-Glu-Gly and the hexapeptide $(Ala)_6$ in cells of *L. monocytogenes* was investigated. Significant uptake rates of both peptides (final concentration, 0.3 mM) were detected in cells incubated in 50 mM potassium phosphate (pH 6.9)-5 mM $MgSO_4$ in the presence of glucose. (Fig. 3). The addition of the potassium ionophore valinomycin (1.5 μM) plus the potassium proton exchanger nigericin (2 μM), which resulted in the complete dissipation of the PMF, inhibited both Val-Leu-Ser-Glu-Gly and $(Ala)_6$ uptake partly. The uptake of the dipeptide Pro-Ala, which has been shown to proceed via a PMF dependent carrier protein (50) was completely abolished upon addition of valinomycin and nigericin (Fig. 3C). At pH 6.0, the uptake rates of both oligopeptides were slightly decreased compared to the uptake rates at pH 6.9. The addition of both valinomycin and nigericin to the assay mixtures at pH 6.0, had a more

dramatic effect on the transport rate of the oligopeptides than at pH 6.9 (about 70% reduction compared to the control; data not shown), which is probably a result of lowering of the internal pH. These experiments show that Val-Leu-Ser-Glu-Gly and $(Ala)_6$ transport can proceed in the absence of a PMF. The nature of the energy source for oligopeptide transport was further investigated by analysis of the effect of the phosphate analog vanadate on Val-Leu-Ser-Glu-Gly and $(Ala)_6$ transport. Uptake of both peptides was completely inhibited in the presence of 0.2 mM vanadate in 50 mM K-HEPES (pH 7.5)-5 mM $MgSO_4$ (Fig. 4).

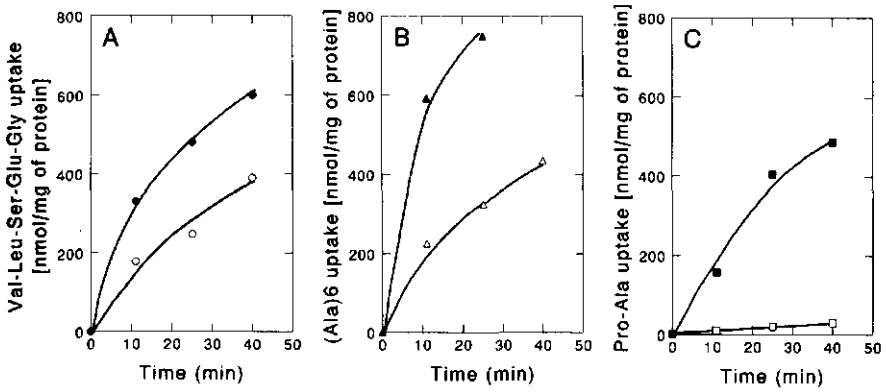

Figure 3. Uptake of peptides in *L. monocytogenes*. Uptake assays with Val-Leu-Ser-Glu-Gly (A), $(Ala)_6$ (B) and Pro-Ala (C) were performed in 50 mM potassium phosphate (pH 6.9) containing 5 mM $MgSO_4$ in glucose-energized cells in the absence (*closed symbols*) and presence (*open symbols*) of nigericin (2 μM) and valinomycin (1.5 μM).

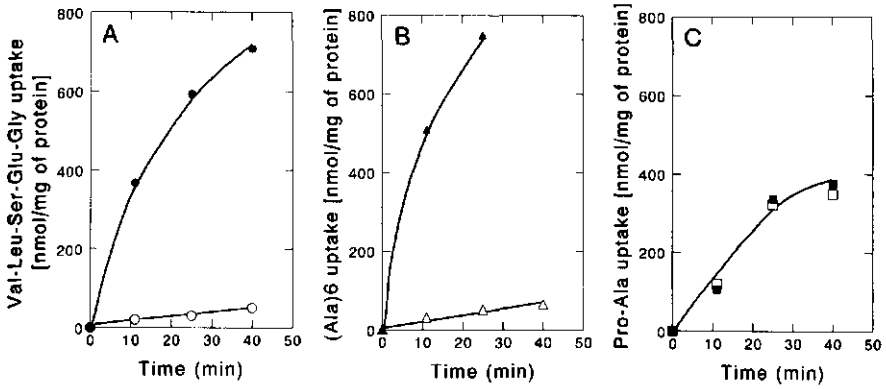

Figure 4. Uptake of peptides in *L. monocytogenes*. Uptake assays with Val-Leu-Ser-Glu-Gly (A), $(Ala)_6$ (B) and Pro-Ala (C) were performed in 50 mM potassium HEPES (pH 7.5) containing 5 mM $MgSO_4$ in glucose-energized cells in the absence (*closed symbols*) and presence (*open symbols*) of vanadate (0.2 mM).

Vanadate had no influence on Pro-Ala uptake, which was expected, since dipeptide transport in *L. monocytogenes* is driven by the PMF (50) and is not affected by ATP directly. The results show that oligopeptide transport in *L. monocytogenes* proceeds via a transport system which is different from the di-tripeptide transport in the organism and that the energy requirement for oligopeptide transport is ATP rather than the PMF.

Effect of proteolytic enzymes and bacteria on growth of *L. monocytogenes*. *L. monocytogenes* fails to grow in DM lacking amino acids or in DM lacking amino acids with ß-casein or Na-caseinate present as the sole source of nitrogen (Fig. 5). The addition of ß-casein or Na-caseinate which had been hydrolyzed with a protease from *B. licheniformis* to DM without amino acids results in a stimulation of growth of *L. monocytogenes*. The stimulation was somewhat more pronounced with hydrolyzed Na-caseinate (containing α, ß and κ casein) than with hydrolyzed ß-casein (Fig. 5).

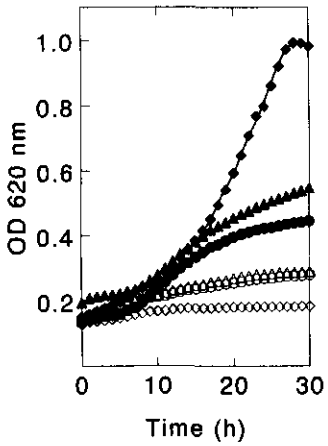

Figure 5. Stimulatory effect of hydrolyzed protein on growth of *L. monocytogenes*. *L. monocytogenes* was grown at 30°C in DM (◆), in DM lacking amino acids (◇), in DM lacking amino acids but with 0.9% (wt/vol) Na-caseinaat (Δ), in DM lacking amino acids but with 0.9% (wt/vol) ß-casein (○), in DM lacking amino acids but with hydrolyzed 0.9% (wt/vol) Na-caseinaat (▲), in DM lacking amino acids but with hydrolyzed 0.9% (wt/vol) ß-casein (●). Protein was hydrolyzed with a protease from *B. licheniformis*. Growth was monitored spectrophotometrically at an OD_{620}.

Growth of *L. monocytogenes* at 20°C in DM with Na-caseinate present as the sole source of nitrogen was stimulated in the presence of *B. cereus* or *P. fragi*. In the control, *L. monocytogenes* increased from 1.0 x 10^6 Colony Forming Units (CFU)/ml to 1.6 x 10^7 CFU/ml in 75 h, whereas with either *B. cereus* or *P. fragi*, *L. monocytogenes* grew to about 1.0 x 10^9 (Fig. 6A). The growth of *B. cereus* or *P. fragi* was not affected by the presence of *L. monocytogenes*, levels amounted to 1.3 x 10^7 and 5.0 x 10^7 CFU/ml, respectively (data not shown). Likewise, *L. monocytogenes* reached high levels (from 5.8 x 10^5 to about 2 x

10^9 CFU/ml at 30°C in 25 h) in DM without amino acids containing Na-caseinate, which had been pregrown with *B. cereus* or *P. fragi* as described in Materials and Methods, whereas in the control *L. monocytogenes* only grew up to 6.3 x 10^6 CFU/ml (Fig 6B).

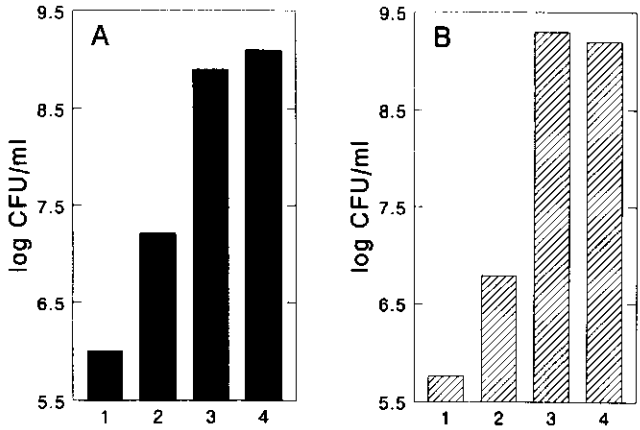

Figure 6. Effect of *B. cereus* and *P. fragi* on growth of *L. monocytogenes*. (A). Colony forming units (CFU) of *L. monocytogenes* in DM with Na-caseinate as the sole of nitrogen at 20°C during 75 h in the absence (*bar* 2) or presence of either *B. cereus* (*bar* 3) or *P. fragi* (*bar* 4). *Bar* 1 represents the inocculation level. (B). CFU of *L. monocytogenes* in DM without amino acids containing Na-caseinate at 30°C for 25 h (*bar* 2), which had been pregrown with *B. cereus* (*bar* 3) or *P. fragi* (*bar* 4). *Bar* 1 represents the inocculation level.

Discussion

In a previous study we showed that *L. monocytogenes* takes up di- and tripeptides via a PMF dependent permease that can supply the pathogen with essential amino acids for growth (50). The present results show that *L. monocytogenes* is, in addition, able to grow on oligopeptides as source of essential amino acids and the experiments reveal that *L. monocytogenes* possesses an oligopeptide transport system, requiring ATP rather than the PMF as the driving force for translocation.

The growth experiments in DM where valine was replaced by valine-containing peptides of varying length suggest that the oligopeptide uptake system transports peptides containing up to eight amino acid residues. The translocation of the peptide seems to be the limiting step for utilization since the valine-containing nona- and decapeptides, that cannot serve as sources of essential amino acids for growth (Fig. 1), were found to be hydrolyzed upon incubation with CE from *L. monocytogenes*. However, since only a limited number of oligopeptides were tested, it cannot be completely excluded that the transportable species can also be longer than eight residues. The oligopeptide permease (Opp) of gram-negative bacteria (*i.e.*, *Escherichia coli* and *Salmonella typhimurium*) transports up to and including hexapeptides. Transport of longer peptides in gram-negative bacteria may be restricted by the upper size exclusion limits of the outer membrane pores rather than the transporter (36). Indeed, in

gram-positive bacteria, the size restriction seems to be more variable. In *Bacillus subtilis*, tri-, tetra- and pentapeptides can be transported via two different oligopeptide transporters (22, 37). *Streptococcus pneumoniae* possesses an oligopeptide permease that functions in the uptake of peptides consisting of two to seven residues (3) and recently, a hexa-heptapeptide permease was identified in *Streptococcus gordonni* (17). *Lactococcus lactis* expresses an Opp that is capable of transporting peptides of four up to eight residues (25, 47). However, recent experiments, in which translocation of oligopeptides formed by the action of the cell wall-bound extracellular proteinase (PrtP) on the natural substrate ß-casein (instead of commercially available peptides) was analyzed, indicate that oligopeptides consisting of up to ten amino acids may be transported by *L. lactis* (26).

All oligopeptide transport systems described to date belong to the family of binding-protein-dependent transport systems that are composed of multiple subunits and use ATP or a related energy-rich, phosphorylated intermediate to drive the peptide uptake (36). Since the mode of energy coupling to the transport of oligopeptides in *L. monocytogenes* resembles that of other ATP-dependent transport systems, *i.e.* the transport is inhibited by the phosphate analog vanadate (12, 25, 38, 49), the Opp of *L. monocytogenes* most likely also belongs to this family of transporters. The partial inhibition of Val-Leu-Ser-Glu-Gly and $(Ala)_6$ uptake as a result of the dissipation of the PMF by adding nigericin plus valinomycin, is probably a secondary effect since PMF dissipation may coincide with changes in internal pH, ATP pools and turgor pressure (1, 25, 49). In contrast, uptake of the dipeptide Pro-Ala in *L. monocytogenes*, which is driven by the PMF (50), was completely inhibited by the combination of the two ionophores whereas vanadate had no effect on Pro-Ala transport (Fig. 3 and 4).

The oligopeptide transport system of *L. monocytogenes* has a relatively high level of activity. The observed uptake rates in cells grown in BHI of Val-Leu-Ser-Glu-Gly and $(Ala)_6$ at pH 6.9 (at an external peptide concentration of 0.3 mM), were approximately 35 and 60 nmol min^{-1} mg of $protein^{-1}$, respectively, whereas Pro-Ala is transported at a rate of about 15 nmol min^{-1} mg of $protein^{-1}$ under the same conditions (Fig. 3). In *L. lactis* cells cultivated in complex broth medium (MRS), tetra-, penta- en hexa-alanine are transported with rates of 2.3, 8.0 and 2.3 nmol min^{-1} mg of $protein^{-1}$, respectively, at an external peptide concentration of 0.5 mM (25). Uptake rates of zwitterionic di- and tripeptides in *L. monocytogenes* and *L. lactis* have been shown to be of the same magnitude (15, 25, 43, 50). Since *L. monocytogenes* cannot utilize proteins as a source of amino acids, the relatively high rates of oligopeptide uptake can be advantageous to *L. monocytogenes* during growth of the organism in foods that are deficient in free amino acids and small peptides but rich in oligopeptides as a result of proteolytic activity of other microorganisms (see also below).

In *L. lactis*, more than ten peptidases displaying different substrate specificities have been identified over the years (26, 33), whereas for *L. monocytogenes* this area of research is almost completely unexploited. In our previous study, we detected N-terminal aminopeptidase activities in CE using lysyl-*p*-nitroanilide, leucyl-*p*-nitroanilide, and alanyl-*p*-nitroanilide, which were between 0.2 and 0.8 nmol min^{-1} mg of $protein^{-1}$. These values are about 50- to 100-fold lower than those reported for lactococci (5, 44, 50). In the present work, release

of nitroanilide could not be demonstrated in CE with the chromogenic substrates alanyl-prolyl-*p*-nitroanilide,glycyl-prolyl-*p*-nitroanilide,acyl-alanyl-alanyl-alanyl-*p*-nitroanilide,and isoleucyl-prolyl-arginyl-*p*-nitroanilide, whereas a peptidase activity of 2 nmol min^{-1} mg of $protein^{-1}$ was found in CE with succinyl-alanyl-alanyl-prolyl-phenylalanyl-*p*-nitroanilide. A similar hydrolysis rate (2 nmol min^{-1} mg of $protein^{-1}$) could be observed with $(Ala)_6$ as a substrate. Tan and Konings (44) reported that the aminopeptidase N (PepN) of *L. lactis*, which shows a high activity towards lysyl-*p*-nitroanilide, had a very low activity towards the chromogenic substrates alanyl-prolyl-*p*-nitroanilide and alanyl-alanyl-alanyl-*p*-nitroanilide. Considering the relatively low aminopeptidase activity found for *L. monocytogenes* compared to that of *L. lactis*, this would validate our present findings. To degrade proline-containing oligopeptides, lactic acid bacteria generally make use of a X-prolyl-dipeptidyl-peptidase (PepXP) (8). Using the chromogenic substrate glycyl-prolyl-*p*-nitroanilide, specific PepXP activities between 85 and 300 nmol min^{-1} mg of $protein^{-1}$ have been recorded for *L. lactis* (7, 20, 31). However, with *L. monocytogenes* no release of nitroanilide was found with glycyl-prolyl-*p*-nitroanilide, suggesting a low level of PepXP-like activity. This in turn would explain the accumulation of particularly proline-containing peptides in cells of *L. monocytogenes* after growth in peptone or in defined medium in the presence of those peptides (4).

Products that have been involved in foodborne listeriosis (raw milk, raw meat and minimally processed vegetables) generally harbor diverse populations of microorganisms. These include, besides *L. monocytogenes*, proteolytic microbes like *Pseudomonas* spp., *Bacillus* spp., and lactic acid bacteria. In addition, *L. monocytogenes* and *Pseudomonas* spp. appear frequently in pasteurized dairy products as post-processing contaminants, whereas spores of bacilli survive pasteurization (2, 9, 11, 16, 23, 30, 34). In this study, *P. fragi* DSM 3456 and *B. cereus* VC2 were shown to enhance the growth of *L. monocytogenes* in a medium with casein present as the sole amino acid source (Fig. 6). In addition, hydrolysis of casein using purified *B. licheniformis* protease, increased the growth of *L. monocytogenes* (Fig. 5). These results suggest that proteolysis of the milk protein casein can provide stimulatory factors (*i.e.* large and small peptides and amino acids) for the growth of *L. monocytogenes*. The oligopeptide transport system of *L. monocytogenes* described herein, might have been of importance in the utilization of these breakdown products. Taking into account the psychrotrophic nature of *L. monocytogenes*, especially psychrotrophic species of *Pseudomonas* and *Bacillus* may stimulate the growth of *L. monocytogenes* during refrigerated storage of foods (9, 45). In fermented dairy products such as cheese casein is degraded by the cell envelope-located proteinase (PrtP) of lactococci, which results in the formation of more than 100 different peptides ranging from four to ten residues (18). To date no significant release of amino acids or di- and tripeptides has been observed in hydrolysates formed by various lactococcal proteinases (26). In the initial fermentation phase (high pH, low level of lactic acid), the oligopeptide transport system may be crucial to supply *L. monocytogenes* with essential amino acids allowing it to grow. Recently, *L. monocytogenes* has been shown to exhibit adaptive acid tolerance response (ATR) following adaptation to mildly acidic conditions, which is capable of protecting cells from normally lethal acid stress (24, 35). The oligopeptide transport system of the pathogen in combination with the

development of ATR might therefore result in increased numbers of *L. monocytogenes* in fermented milk products (14).

The proteolytic system of *L. monocytogenes* is schematically summarized in Fig. 7. The lack of an active protease and its inability to synthesize all the amino acids from inorganic ammonium salts, make the pathogen dependent on the proteolytic activity of other microorganisms present in the food for the hydrolysis of food proteins. In addition, indigenous proteinases might to some extent contribute to the hydrolysis of food proteins (6, 13, 27). For an efficient utilization of the hydrolyzed products, *L. monocytogenes* is equipped with amino acid transporters (21, 48), a PMF-driven di-tripeptide carrier (50) and an ATP-dependent oligopeptide uptake system that most likely is composed of multiple subunits, which is capable of transporting peptides up to at least eight residues. The acccumulated peptides are subsequently degraded by peptidases that are located intracellularly in order to supply *L. monocytogenes* with the amino acids necessary for protein biosynthesis.

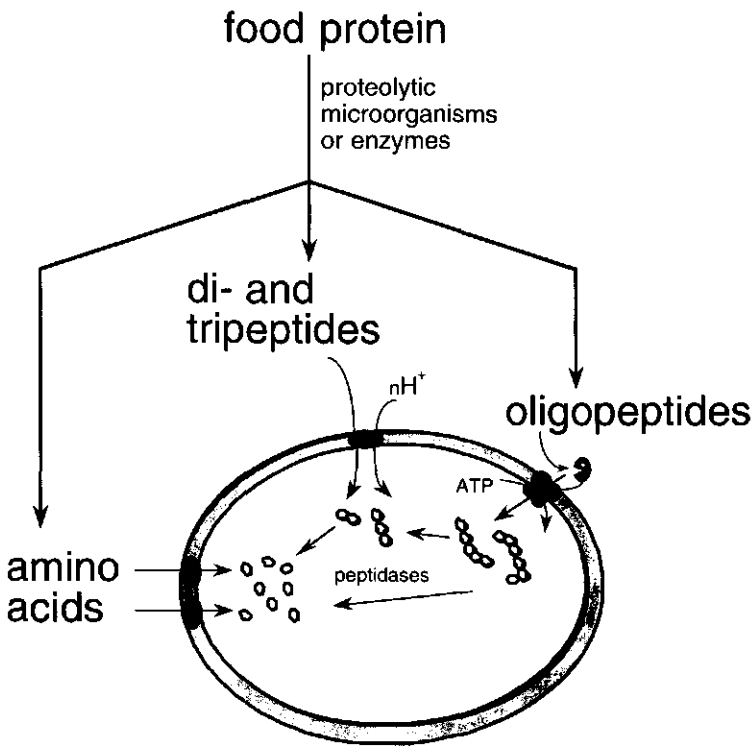

Figure 7. Model for the proteolytic system of *L. monocytogenes*. See text for explanation.

Acknowledgements

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4

The role of peptide metabolism in the growth of *Listeria monocytogenes* ATCC 23074 at high osmolarity

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Abstract

The growth of *Listeria monocytogenes* ATCC 23074 in defined medium is sensitive to high osmolarity when compared with its growth in complex media, such as brain heart infusion (BHI). The two major contributors to this difference in growth rate are the availability in BHI of the osmoprotectant betaine and peptides. Peptone plays two major roles: firstly as a nutritional supplement for protein synthesis, and secondly as a source of amino acids and peptides that serve as a mechanism of maintaining turgor. In the presence of peptone the total amino acid pool at high osmolarity is substantial and even in the presence of betaine the amino acid pool makes a major contribution to turgor maintenance. At high osmolarity there is a general increase in amino acid pools, with particularly substantial pools of glutamate, aspartate, proline, hydroxyproline and glycine. Peptides are also accumulated by cells from the peptone supplied in the medium. Glycine-containing peptides are accumulated in the cytoplasm under all conditions. Specific glycine- and proline-containing peptides stimulate growth at high osmolarity. The peptide prolyl-hydroxyproline accumulates in cells to high levels in response to growth at high osmolarity, and the pools of the derived amino acids also show a dependence on the external osmotic pressure. However, proline only confers significant osmoprotection when supplied as peptides. The significance of these data in the context of the occurence of *L. monocytogenes* in foods with high peptide content is discussed.

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Introduction

Listeria monocytogenes is a Gram-positive intracellular parasite responsible for severe opportunistic infections in humans and other animals. The ability of *L. monocytogenes* to survive a wide range of environmental conditions including relatively high salt concentrations (10%, wt/vol, NaCl) and low temperatures allows this bacterium to resist traditional food preservation methods and to persist under conditions where the growth of other microorganisms is inhibited (12, 15). This persistence is graphically illustrated by an outbreak of listeric septicaemia that has been linked to consumption of contaminated salted mushrooms stored in 7.5% NaCl at low temperature for 5 months (7).

The adaptation of bacteria to water stress involves the intracellular accumulation of certain solutes, which increase the intracellular water activity and allow the bacterial cells to survive high or fluctuating osmolarities, desiccation or freezing. These solutes are known as compatible solutes because they can be accumulated at high concentrations without inhibiting vital cellular processes. The term osmoprotectants is also used to describe their ability to overcome growth inhibition due to osmotic stress (3, 4, 19). The main osmoprotectants in moderate halophilic bacteria are betaine and proline, for which a number of transport mechanisms have been described in *Escherichia coli*, *Salmonella typhimurium* (2), in the Gram-positive bacterium *Staphylococcus aureus* (14) and recently also in *L. monocytogenes* (9). In *E. coli* and *S. aureus* betaine can be synthesized from choline when it is present in the medium (8, 17).

The aim of this study was to investigate the physiological response of *L. monocytogenes* to osmotic stress by identifying the osmoprotectants that allow the growth of this food pathogen at high salt concentrations. We demonstrate that the growth of *L. monocytogenes* at high osmolarity is strongly stimulated by the addition of peptone to the medium and that this has a comparable effect on growth rate to that of the compatible solute betaine. Proline-containing peptides also stimulate growh at high osmolarity and can substitute for betaine. We demonstrate that *L. monocytogenes* accumulates high levels of certain peptides during growth at both low and high osmolarity and that these peptides contribute to osmoregulation.

Materials and Methods

Bacterial strain and growth conditions. *L. monocytogenes* ATCC 23074 (serotype 4b) was supplied by Professor G.S.A.B. Stewart, University of Nottingham, UK, and maintained in coryneform medium agar plates at 4°C (13), for a maximum of two weeks. New plates were prepared by streaking out an aliquot from the glycerol stock, which was kept at -70°C for long-term storage. Growth experiments were carried out at 30°C in brain heart infusion (BHI) or in a defined medium.

Defined medium. The chemically defined medium (DM) prepared for *L. monocytogenes* ATCC 23074 was a modification from Patchett *et al.* (13) and contained (g l^{-1}): $K_2HPO_4.3H_2O$, 10.5; $NaH_2PO_4.2H_2O$, 3.1; NH_4Cl, 1.0; $MgSO_4.7H_2O$, 0.4. The following amino acids were added to the growth medium (g l^{-1}): L-leucine, 0.1; L-isoleucine, 0.1; L-valine, 0.1; L-methionine, 0.1; L-tryptophan, 0.1; L-cysteine, 0.1; L-histidine.HCl, 0.1; L-arginine.HCl, 0.2; L-glutamine, 0.6. A 100-fold concentrated stock solution of vitamins was made up according to Trivett and Meyer (18): 5 mg α-lipoic acid was dissolved in 200 ml 70% (vol/vol) ethanol and 2 ml of this solution was added to

125 ml 95% (vol/vol) ethanol containing 5 mg biotin, 50 mg thiamin.HCl and 50 mg riboflavin. The solution was made up to a final volume of 500 ml with distilled water and was then filter-sterilized. A trace metal solution (modified from Harwood and Archibald (6)) was prepared as a 100-fold concentrated stock. The stock solution contained (g l^{-1}): $CaCl_2.2H_2O$, 0.275; $FeCl_3.6H_2O$, 0.675; nitrilotriacetic acid, 6.75; NaOH, 3.375; $ZnCl_2$, 0.085; $CuCl_2$, 0.0215; $CoCl_2$, 0.03; $Na_2MoO_4.2H_2O$, 0.03. The trace element solution was made by adding the components listed above sequentially to distilled water such that each was dissolved before the next was added. The calculated osmolality of DM was approximately 260 mosM.

Growth studies. Single colonies of *L. monocytogenes* were used as inoculum for overnight growth either in DM under limiting glucose conditions (0.05%, wt/vol) or in BHI, at 30°C with agitation (400 rpm) in a shaker incubator (New Brunswick). Cells were subsequently supplemented with glucose (0.5%, wt/vol) and allowed to grow to exponential phase, prior to inoculation into fresh media (25 ml) to a starting OD_{600} of 0.05-0.10. Cell growth was monitored spectrophotometrically (Ultrospec 4050, LKB, Biochrom) by measuring OD_{600} of 1 ml samples taken every hour.

Fractionation of peptone. Peptone was fractionated according to charge using an anion-(Bio-Rad AG 1-X2, acetate form) and a cation-exchanger (Bio-Rad AG 50W). Two columns were poured and equilibrated with 25 mM MOPS pH 7.04. Peptone ((5%, wt/vol) in 25 mM MOPS, pH 7.04) was passed through the anion-exchange column and the first fraction collected contained the neutral and positively charged residues. The negative residues, bound to the resin, were eluted with 0.5 M NaCl in 25 mM MOPS, pH 7.04. The neutral and positively charged residues were separated by the cation-exchange column, with the neutral residues being collected first and the positively charged residues eluted with NaCl as before.

Amino acid analysis. Cells were harvested at late exponential phase by filtration through a Whatman membrane filter (0.45 μm), under vacuum. Filters were washed twice immediately, with a slightly hypertonic NaCl solution. Intracellular solutes were extracted from the cells with 0.1% trifluoroacetic acid (TFA), on ice for at least 30 min, and samples were kept at -20°C for further analysis. Aliquots (200 μl) were filtered through 10 kDa molecular mass cut-off filters followed by centrifugation. Norleucine (250 pmol) was added as an internal standard to 20 μl samples, which were loaded onto an Applied Biosystems 420H amino acid analyser equipped with automatic hydrolysis and derivatization. Analyses were carried out using hydrolysis and derivatization for normal peptides and derivatization only for free amino acids. The phenylthiocarbamyl (PTC) amino acids generated were identified on-line, using a 130A separation system employing a C18 reverse-phase narrow-bore cartridge 200 mm x 2.1 mm at a temperature of 34°C. Eluents A and B were 50 mM sodium acetate buffer, pH 5.44, and 70% acetonitrile/30 mM sodium acetate buffer, respectively, with a gradient of 7% B at time 0; 16% B at 5 min; 30% B at 10 min and 54% B at 20 min at a flow rate of 300 μl min^{-1}. The PTC amino acids thus separated were identified and quantified by the UV absorbance detector, set at 254 nm, and a Spectra Physics WINner on Windows Integration System set at an attenuation of 32 mV.

Measurement of betaine accumulation during growth. This was determined by the method of Koo and Booth (10). Cultures were grown in DM containing 0.625 M NaCl, 0.5% (wt/vol) peptone and 200 μM betaine. When the cells had established exponential growth (OD_{600} = 0.2) an aliquot (2.5 ml) was transferred to a wide test tube (2.5 cm diameter) containing 10 nCi (370 Bq) of radioactive betaine and the incubation continued. When growth in the control flask had reached OD_{600} 0.4, the tube was removed from the incubator and the following manipulations undertaken. The OD_{600} of the culture in the tube was recorded; three 0.5 ml samples were taken and filtered through glass-fibre

filters and washed with 3 ml growth medium of the same composition but lacking betaine. The filters were then dried and the radioactivity trapped in the cells determined by scintillation counting; 50 μl samples were removed and transferred to filters and dried as standards for the determination of the betaine specific activity.

Protein determination. The relationship between optical density and protein concentration was established by serial dilution of exponential-phase cultures and lysis of the cells of known OD_{600} with 0.1 M NaOH. The protein content was measured by the Folin-Ciocalteu method adapted for microtitre plates using a bovine serum albumin standard. A cell suspension giving a OD_{600} of 1 was equivalent to approximately 170 μg total protein ml^{-1} at low osmolarity and 130 μg total cell protein ml^{-1} at high osmolarity.

Chemicals. The chemicals used were of analytical grade, whenever possible, and supplied by Sigma or BDH, Tryptone, BHI, yeast extract and agar (no. 3) were purchased from Unipath (Oxoid). Peptone type I and peptides were supplied by Sigma. Molecular mass 10 kDa cut-off filters were obtained from Millipore.

Results

Growth of *L. monocytogenes* at high osmolarity. The growth of *L. monocytogenes* at high osmolarity was investigated in BHI and in DM. At low osmolarity the specific growth rates of *L. monocytogenes* were 0.86 $\pm$ 0.08 h^{-1} and 0.46 $\pm$ 0.02 h^{-1} for BHI and DM, respectively. As the osmolarity of BHI cultures was increased there was a marked lag prior to the establishment of a constant exponential growth rate. In contrast, growth in DM at high osmolarity proceeded without a significant lag. In both media the growth rate fell as the osmolarity of the growth medium was increased (Fig. 1). The upper limit for growth was 2.0 M NaCl and 1.0 M NaCl for BHI and DM, respectively. Supplementation of DM, but not BHI, with betaine (1 mM) stimulated growth at moderate osmolarity (0.3-0.7 M NaCl) and extended the upper limit for growth from 1.0 M NaCl to 1.5 M NaCl, but was without effect in low-osmolarity medium. As expected from studies with other organisms the effect of betaine was progressively greater at higher osmolarity (Fig. 1). In contrast proline (1 mM), which serves as an osmoprotectant for many organisms, and choline (1 mM), glycine (1 mM) and betaine aldehyde (0.6 mM), which can serve as precursors of betaine, were without effect on the growth of *L. monocytogenes* (data not shown). Recently, 10 mM proline has been found to confer significant osmoprotection on *L. monocytogenes* up to 0.5 M NaCl (1).

Growth stimulation by peptone. The osmotic tolerance of *L. monocytogenes* was clearly greater in complex medium than in DM and the components of BHI that were responsible for this effect were investigated. The addition of peptone, a major component of BHI (0.5%, wt/vol), to low osmolarity DM caused a 1.5-fold stimulation of growth, as expected for nutrient supplementation (Table 1). However, addition of peptone to the higher osmolarity medium DMS (DM plus 0.625 M NaCl) stimulated growth threefold (Table 1). The addition of betaine was additive, with the effect of peptone leading to more that fourfold stimulation of growth rate (Table 1). The growth rate achieved by *L. monocytogenes* in DMS plus peptone and betaine was identical to that observed in BHI medium at 0.625 M NaCl. Thus,

it appears that *L. monocytogenes* requires nutritional supplementation as well as osmoprotectants in order to achieve its characteristic salt tolerance.

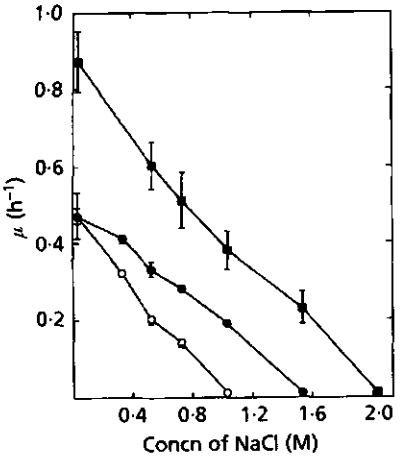

Figure 1. Growth rate of *L. monocytogenes* as a function of NaCl added to the medium. An overnight culture of *L. monocytogenes* was cultured on the appropriate medium as described in Methods and the specific growth rate (μ) was determined during exponential growth. ○, DM; ●, DMB (DM supplemented with 1 mM betaine); ■, brain heart infusion.

Table 1. Growth stimulation by peptone and betaine. Cells were grown overnight as described in Fig. 1 and inoculated into fresh growth medium. The specific growth rate was determined from the exponential phase of growth by taking samples at regular intervals and plotting OD_{600} versus time. Data represent the mean and the standard deviation of (*n*) experiments.

Medium	Addition(s)		Specific growth rate (h^{-1}) in medium containing:	
	Peptone (0.5%, wt/vol)	Betaine (1 mM)	No NaCl	0.625 M NaCl
DM	-	-	0.46 ± 0.02 (*5*)	0.12 ± 0.02 (*5*)
	+	+	0.69 ± 0.01 (*2*)	0.51 ± 0.01 (*2*)
	+	-	0.69 ± 0.01 (*2*)	0.39 ± 0.02 (*3*)
BHI	-	-	0.86 ± 0.08 (*2*)	0.50 ± 0.07 (*2*)
	-	+	0.86 ± 0.08 (*2*)	0.50 ± 0.07 (*2*)

The peptone used in this study is a complex mixture of peptides and amino acids derived from hydrolysis of animal proteins. Further analysis of the growth stimulation provided by peptone was achieved by anion- and cation-exchange chromatography to generate neutral,

acidic and basic peptide fractions. A 5% (wt/vol) solution of peptone was fractionated as described in Methods and each of the three major classes of peptides investigated for their ability to stimulate growth in DMS and DM. In DM the growth rate was stimulated by the neutral fraction of peptides, but the lag time to the establishment of exponential growth appeared to be reduced by all three fractions. Specific growth rates of 0.49, 0.69, 0.53 and 0.52 h^{-1} were observed with DM, DM plus neutral peptides, DM plus anionic peptides and DM plus cationic peptides, respectively. In DMS all three peptide fractions stimulated growth, but only the neutral fraction gave an initial growth rate that was identical to that observed with unfractionated peptone (specific growth rates of 0.42 and 0.41 h^{-1}, for complete peptone and neutral peptide fraction, respectively). Thus, the major growth stimulation derives from the neutral fraction of peptides.

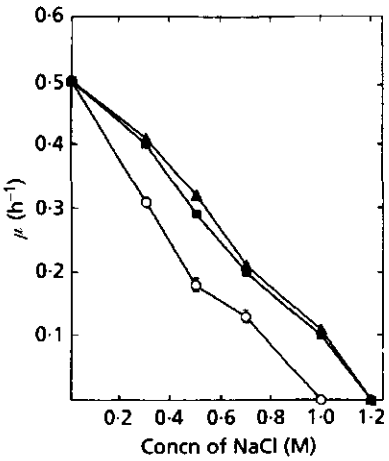

Figure 2. Growth rate of *L. monocytogenes* in defined medium (DM) supplemented with peptides as a function of NaCl added to the medium. Details of growth were as described in Figure 1 and Methods. ○, DM; ▲, DM supplemented with 1 mM prolyl-glycyl-glycine; ■, DM with 1 mM prolyl-hydroxyproline.

The growth stimulation in DMS of a range of proline-containing peptides was investigated. Prolyl-glycyl-glycine (PGG) and prolyl-hydroxyproline (PHP) strongly stimulated growth (Fig. 2) and prolyl-glycine (PG) also stimulated growth, but to a lesser extent (data not shown). A mixture of proline and glycine (1 mM), added as free amino acids, had no effect on the growth rate at either low or high osmolarity and we were unable to detect transport of [^{14}C]proline (data not shown). In DM proline-containing peptides had no effect on the growth rate (Fig. 2). Growth stimulation in DMS was similar for the tripeptide PGG and the dipeptide PHP and extended the growth limit in DMS to approximately 1.2 M NaCl (Fig. 2), which is consistent with a role for these peptides as generators of osmoprotectants. Additive effects on growth rate in DMS were seen with either a mixture of peptides (PGG plus PHP) or supplementation with both PGG and betaine (not shown).

Table 2. Amino acid pools of *L. monocytogenes* cells grown under different regimes. Cells were grown overnight as described in Figure 1 and inoculated into fresh growth medium. The specific growth rate was determined from the exponential phase of growth as described in Table 1. When the cells had reached late exponential phase, samples were taken for amino acid analysis as described in Methods. Data are expressed as nmol (mg cell protein)$^{-1}$ and are shown as the mean and standard deviation of duplicate estimates of *n* experiments.

Amino acid	Size of amino acid pool [nmol (mg cell protein)$^{-1}$] of cells grown in:				
	DM ($n=5$)	DMP ($n=2$)	DMS ($n=4$)	DMSP ($n=3$)	DMSPB ($n=2$)
Specific growth rate (h^{-1})	0.46±0.03	0.69±0.00	0.13±0.01	0.39±0.02	0.51±0.01
Asp	125±25	86±31	180±24	152±25	55±9
Hyp	0±0	75±26	0±0	348±45	183±22
Glu	1125±60	612±13	1766±187	1346±59	897±161
Asp	0±0	0±0	1±2	2±2	1±1
Ser	8±2	18±5	8±5	75±25	21±18
Gln	72±56	29±7	853±169	210±100	42±24
Gly	18±4	73±45	43±64	436±20	167±13
His	6±4	5±2	21±4	1±2	4±2
Arg	6±4	10±3	6±2	8±8	12±8
Thr	7±4	14±2	5±3	36±32	15±10
Ala	22±18	85±19	358±141	233±34	70±32
Pro	14±16	24±21	69±12	170±10	65±5
Tyr	1±2	1±2	0±0	2±2	2±2
Val	21±19	21±1	60±8	57±16	43±15
Met	3±5	5±2	12±2	15±8	9±14
Ile	26±4	10±1	35±12	25±1	21±10
Leu	26±3	24±5	44±7	66±25	38±9
Phe	4±5	1±2	1±2	5±5	5±2
Trp	1±2	1±2	2±3	2±3	1±1
Lys	5±3	9±3	22±17	9±4	3±2
Total	1592±221	1121±46	3560±372	3184±185	1665±296

Amino acid and peptide accumulation by osmotically stressed cells. The amino acid pools of cells are the consequence of several conflicting processes: synthesis, transport, utilization for protein synthesis, excretion, deamination and metabolism, and peptidase

activity (when peptides are present in the medium). Analysis of the amino acid pools can only reveal the resultant of these activities, and alterations in the pool may reflect changes in any of these parameters. The amino acid pools of *L. monocytogenes* were investigated under the conditions used for growth analysis (above). Supplementation of DMS with peptone (DMSP medium) caused small changes in the pool sizes of many amino acids, but significant increases in the pools of proline, glycine, alanine, hydroxyproline, aspartate, glutamate and glutamine (Table 2). The pools of glutamate, glutamine and aspartate were found to be inversely correlated with the growth rate (Fig. 3). When the growth rate was stimulated by the addition of betaine to DMSP (*i.e.* DMSPB; Table 2) the pools of most amino acids were reduced. A similar effect of betaine has been observed for cells growing in peptone-free medium containing high salt (0.625 M NaCl; data not shown). Consequently, in analysing the changes in amino acid pools during growth under conditions of osmotic stress it is necessary to take into consideration alterations of other parameters, such as growth rate. Thus, it may not always be possible to attribute changes in amino acid pools to a single physiological mechanism.

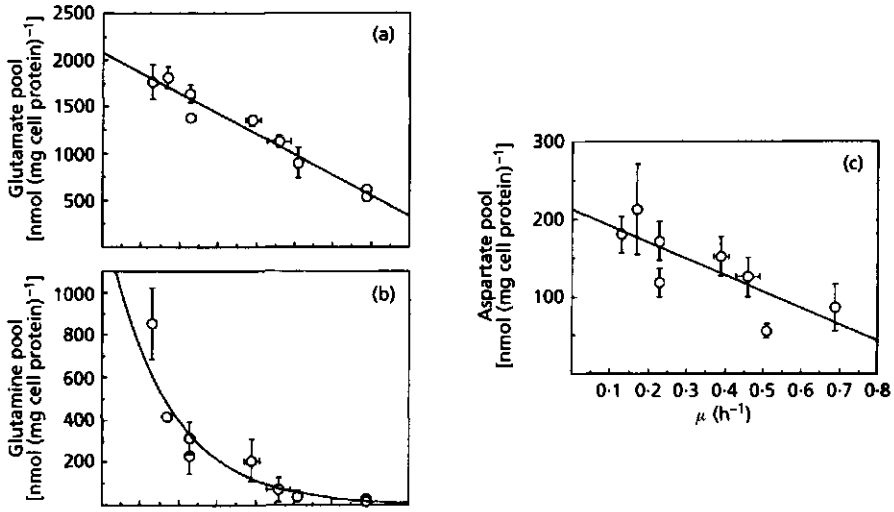

Figure 3. Effect of growth rate on pools of (a) glutamate, (b) glutamine and (c) aspartate. Cells were grown in DM with additions of peptone, specific peptides, betaine and sodium chloride and the specific growth rate (μ) was determined. When the cells were in late exponential phase samples were taken and the amino acid pools determined as described in Methods.

Supplementation of DMS with the peptides PHP, PG and PGG provoked the accumulation of large pools for the amino acids corresponding to the peptide (Table 3). In general it was found that glycine pools derived from PGG or PG, and proline pools derived from PHP, were lower than predicted from the stoichiometry of the peptides, suggesting that these amino acids were either lost from the cell or were more rapidly metabolized.

Table 3. Amino acid and peptide pools of *L. monocytogenes* cells. Cells were grown in DM containing 0.625 M NaCl and the appropriate peptide. When the cells were established in exponential growth samples were taken and analysed for amino acid and peptide content as described in Methods.

Peptide supplied[a]	Size of pool [nmol (mg cell protein)$^{-1}$] of:					
	Free amino acids			Peptides[b]		
	Gly	Pro	HydroxyPro	PHP	PGG	GG
PHP	13±18	312±32	538±58	724	nil	nil
PGG	942±28	707±238	nil	nil	228	146
PHP + PGG	582±5	568±55	205±25	592	263	161

[a] Peptides were supplied at a final concentration of 1 mM.

[b] Peptide content is calculated from the amino acid content of the sample after acid hydrolysis corrected for the content prior to hydrolysis. Standard deviations are omitted since this is a calculated value rather than a direct measurement.

It is known that peptides can be accumulated as contributors to the restoration of turgor pressure (5, 11, 16). Usually such peptides are derived by synthesis from the free amino acids, such as glutamate and glutamine. We sought to determine whether *L. monocytogenes* accumulated free peptides when grown with peptone or with specific peptides and what role such peptide accumulation might play in osmoregulation. Cell lysates were analysed for free amino acids and peptides as described in Methods. Cells grown at either low osmolarity (DMP) or high osmolarity (DMSP) accumulated similar pools of peptides, composed principally of glycine, proline and hydroxyproline (Fig. 4). Similarly when cells were incubated with the individual peptides PGG, PHP and PG, a substantial pool of the unhydrolysed peptide was maintained for PGG and PHP, but not for PG (Fig. 5). For the tripeptide PGG the amino acid pools after acid hydrolysis are consistent with the accumulation of both PGG and glycyl-glycine (GG) derived from PGG by peptidase action. Cells incubated in DMS with PGG and PHP had reduced pools of PHP (approximately 18% reduction) and increased PGG and GG pools (13% and 10%, respectively for PGG and GG) when compared with cells incubated with a single peptide (Table 3). Correspondingly it was observed that the pool of hydroxyproline was also reduced relative to cells grown with PHP alone (Table 3). Thus, when the peptides are in competition for transport systems the tripeptides may be preferred substrates.

The intracellular concentrations of the peptides can be very high, for example peptide pools in cells grown in DMS plus PHP and PGG were as much as 1 μmol (mg cell protein)$^{-1}$, which would be approximately 330 mM based on a cytoplasmic volume of approximately 3 μl (mg cell protein)$^{-1}$ (13). These pools are similar to the total pools of free amino acids (450

mM; Table 3) and consequently both contribute substantially to osmoregulation. For comparison we determined the intracelllular concentration of betaine during mid-exponential growth. Cells were cultured in DMSP containing 200 μM [$^{14}CH_3$]betaine (specific activity 0.02 μCi μmol^{-1}, 0.74 kBq μmol^{-1}) and the internal pool of betaine determined as described previously (10). Cells were found to have accumulated betaine to 2.5 $\pm$ 0.4 μmol (mg cell protein)$^{-1}$, which corresponds to an intracellular concentration of approximately 800 mM. This accumulation of betaine was accompanied by a decrease of the total amino acid pool of approximately 50% from 1 M to 0.55 M (Table 2). Patchett *et al.* (13) recorded that small increases in the potassium pool accompanied growth at higher osmolarity. Taking these data with our own it is clear that under a variety of nutritional conditions, provided by varying the peptide supply, cells are attaining an internal osmotic pressure in the range 1 to 1.5 osM. Thus, in the absence of betaine the accumulation of peptides and amino acids can be used by *L. monocytogenes* to establish an internal osmotic pressure close to that observed when betaine is present.

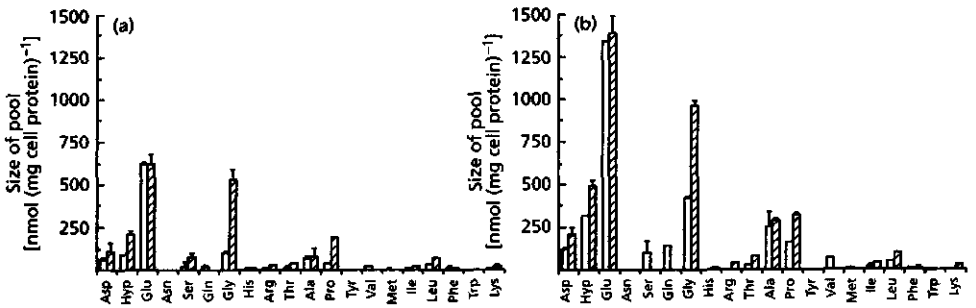

Figure 4. Intracellular pools of amino acids in *L. monocytogenes* grown in the presence of peptone, at low and high osmolarity. The intracellular pools of amino acids were determined as described in Methods. The analysis of amino acids does not measure the contribution of peptides to the cellular pools. Peptide pools were obtained by hydrolysis of the samples followed by re-analysis of the pools as described in Methods. (a) DMP (DM supplemented with 0.5% (wt/vol), peptone); (b) DMSP (DMP with 0.625 M NaCl). *Open bars*, free amino acids in the samples; *hatched bars*, total concentration of amino acids after hydrolysis.

The possible role of the peptides in osmoprotection was investigated further by determining the relative amino acid and peptide pools in cells grown with the peptide PHP in medium of elevated osmolarity (DMS). PHP was chosen because this peptide is derived from collagen and would be expected to be a natural component of many partially hydrolysed meat products. The cytoplasmic PHP pools increased as the osmolarity was raised and this was paralleled by the pools of hydroxyproline and proline (Fig. 6). Above 0.7 M salt the pools of both peptide an free amino acid declined. These data show that physiological changes are taking place as the osmolarity is raised, leading to altered pools of the amino acid and peptide. Clearly, such accumulation of osmotically-active solutes will enable the cell

to compensate for the change in the external osmolarity. These data are consistent with osmotic stimulation of peptide transport. Further the data point to controlled accumulation of proline and hydroxyproline. Thus, the accumulation of peptides may be a mechanism of osmoadaptation in *L. monocytogenes*.

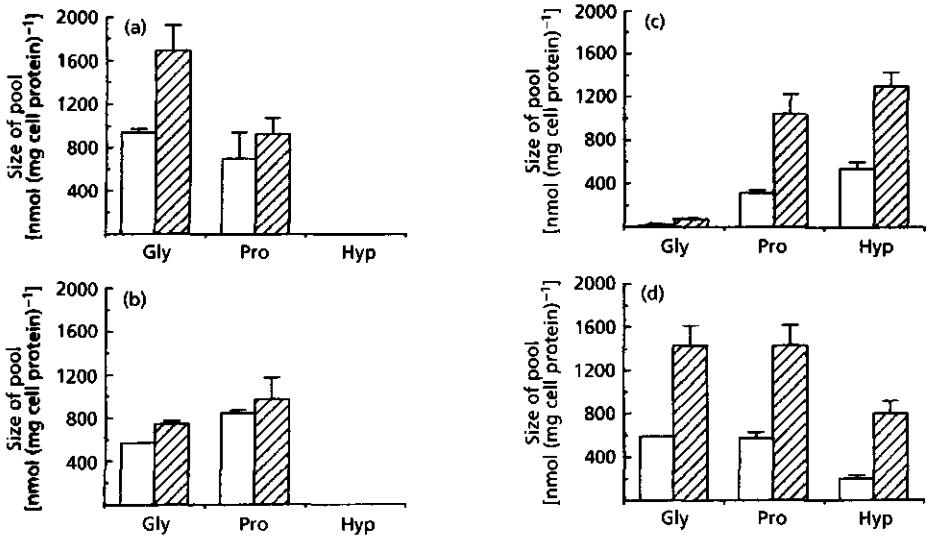

Figure 5. Intracellular pools of glycine, proline and hydroxyproline in *L. monocytogenes* grown in the presence of peptides, at high osmolarity. Cultures were grown to exponential phase on DM containing the peptide(s) indicated and samples were taken for amino acid analysis. Samples were also subjected to acid hydrolysis (see Methods) to analyse the peptide fraction in the cells. (a) DMS (DM containing 0.625 M NaCl) supplemented with 1 mM prolyl-glycyl-glycine (PGG), (b) DMS with 1 mM prolyl-glycine (PG), (c) DMS with 1 mM prolyl-hydroxyproline (PHP), (d) DMS with 1 mM PGG and 1 mM PHP. *Open bars*, free amino acids in the samples; *hatched bars*, total concentration of amino acids after hydrolysis.

Discussion

The data presented in this paper demonstrate that the growth of *L. monocytogenes* is strongly stimulated by peptone. Peptone stimulates bacterial growth through the provision of amino acids that either can be utilized directly for protein synthesis or can be oxidized to provide ATP. The primary role of peptone as a nutrient source is consistent with the largely unchanged amino acid pools found in cells growing at low osmolarity in the presence and absence of peptone. Metabolism of amino acids can also occur to provide carbon skeletons entering the tricarboxylic acid cycle. However, oxidative metabolism plays only a minor role in energy transduction and, consequently, only those amino acids able to give rise to ATP by substrate-level phosphorylation would be important in energy generation. *L. monocytogenes* does not grow with peptides as sole carbon source (unpublished data) and thus, the likely primary role of the peptides during growth at low osmolarity is the provision

of amino acids for protein synthesis. Recently, a di- and tripeptide transport system was shown to be able to supply *L. monocytogenes* with essential amino acids for growth (see Chapter 2).

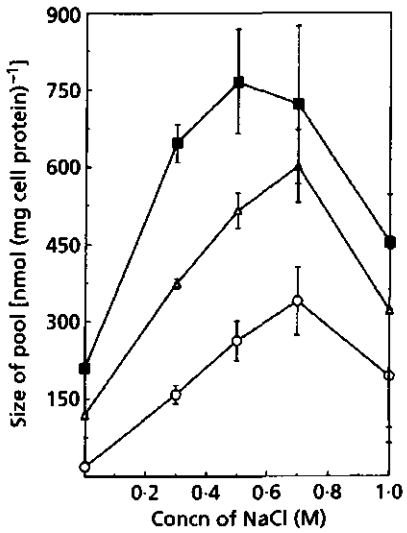

Figure 6. Cytoplasmic pools in *L. monocytogenes* grown in the presence of 1 mM prolyl-hydroxyproline at different NaCl concentrations. *L. monocytogenes* was grown to exponential phase on DM containing PHP (1 mM) and the concentration of NaCl indicated. Samples were taken for amino acid analysis and duplicate samples were treated by acid hydrolysis to analyse the peptide accumulation in the cells. ○, proline; Δ, hydroxyproline; ■, prolyl-hydroxyproline.

Analysis of the role of peptone during growth at high osmolarity is more complex. Growth stimulation is a product of both nutritional supplementation and osmoadaptation. The pools of amino acids and peptides accumulated in cells grown at high salt are similar to those detected when the organism is supplied with betaine and peptone. However, the specific growth rate achieved in the presence of both peptone and betaine is higher than that achieved with either peptone or betaine alone. This simultaneous presence of both betaine and peptone leads to the salt tolerance for which *L. monocytogenes* is renowned and gives specific growth rates similar to those achieved in brain heart infusion.

Peptide accumulation during osmoregulation has previously been noted in a variety of species but these have usually been synthesized *de novo*, rather than accumulated from peptone (5, 11, 16). The data presented here clearly demonstrate that cells grown with peptone accumulate substantial pools of peptides from the growth medium and that these pools can contribute to osmoregulation. With individual peptides, such as PHP, it is clear that the peptide pool is affected by the osmotic pressure of the medium in a manner consistent with a contribution to osmoregulation.

In contrast to the situation for peptides there are substantial increases in the pools of free amino acids at high osmolarity. The fate of peptides in the cell is determined by the relative

balance of influx and efflux via specific transport systems and the rate of hydrolysis of the peptide in the cytoplasm. In this study it is clear that for single peptides the balance of transport and hydrolysis is dependent upon the identity of the amino acids constituting the peptide; *e.g.* for prolyl-glycine (and also the tripeptide alanyl-alanyl-alanine; data not shown) the peptidase activity is able to match the transport capacity of the cell, resulting in little or no accumulation of the free peptide. In contrast prolyl-glycyl-glycine, which differs from PG by a single extra glycine residue, is not readily broken down and significant accumulation of glycyl-glycine occurs. This clearly parallels the situation with peptone since there are substantial pools of glycine-containing peptides in cells grown with peptone. Given the prevalence of prolyl-glycine and hydroxyprolyl-glycine linkages in collagen, the accumulation of this type of peptides by cells may have an important bearing on the growth of *L. monocytogenes* in pâté.

Gram-positive organisms are often considered to sustain very high turgor pressures. Our analysis suggests that this may not be true for *L. monocytogenes* ATCC 23074. An approximation of the osmolarity of the cytoplasm can be obtained from the analysis of the amino acid pools, betaine accumulation and pools of potassium (13), since recent NMR studies on *L. monocytogenes* found no evidence for any endogenously synthesized osmoprotectant (9). In the case of the strain of *L. monocytogenes* studied in this work the calculated internal osmolarity in cells grown in DM is quite low, but is above that of the external osmolality: approximately 400 - 700 mosM in cells compared with 260 mosM in the medium. In contrast, cells growing at high osmolarity in the presence of peptides and betaine (DMSPB) exhibit pools that total approximately 1800 mosM compared with an external osmolality of 1300-1500 mosM (calculated from Table 2 and data for potassium pools and cell volume from reference 13). In the absence of betaine the calculated cytoplasmic osmolarity is somewhat lower (approximately 1400 mosM). Thus these data suggest that the turgor pressure of *L. monocytogenes* ATCC 23074 is of similar magnitude to that calculated for *E. coli*. During growth at high osmolarity the turgor remains relatively constant in the presence of betaine but may decline when the sole source of compatible solutes is the peptone of the growth medium. These data establish for the first time that when this organism is cultured on complex medium, which is similar to their natural habitat as a food-borne pathogen, the accumulation of amino acids and peptides from the environment can substitute for conventional compatible solutes.

Acknowledgements

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Introduction

Epidemics of food-borne listeriosis with high fatality rates (about 25%) have resulted in concern about the incidence and control of *Listeria monocytogenes* in the food supply and environment. An important factor enabling the organism to survive and grow in foods is its ability to grow in the presence of salt concentrations up to 10% (16). Understanding of the process of osmotic adaptation in *L. monocytogenes* could indicate mechanisms for controlling growth of this human pathogen in low-water-activity foods. The survival of bacteria at low water activity generally runs parallel with their ability to accumulate compatible solutes (12, 22). These solutes may be taken up from the surrounding medium, or they may be synthesized *de novo* by the microorganism. Compatible solutes can be accumulated to high concentrations without inhibitory effects on enzymatic activity or adverse effects on macromolecules (51).

The adaptability of *L. monocytogenes* to osmotic stress resembles that of many other bacteria and appears to depend on the ability of the organism to accumulate betaine and amino acids (7, 29, 35). In addition, it was shown that glycine- and proline-containing peptides stimulate growth of this bacterium at high osmotic strength (5). Betaine (*N,N,N*-trimethylglycine) is present at high concentrations in sugar beets and other foods of plant origin (6), whereas in certain food products increased amounts of amino acids and peptides are available as a consequence of proteolytic activity of other bacteria present in this food. Recently, our laboratory has shown that exogenously supplied carnitine (ß-hydroxy-L-τ-*N*-trimethyl aminobutyrate) can contribute significantly to growth of *L. monocytogenes* at high osmolarity (7). L-carnitine is biosynthesized by mammals and eukaryotic microorganisms from the amino acid L-lysine (28), where it serves as an essential factor in the transport of fatty acids, through the inner mitochondrial membrane (8). Consequently, the carnitine concentration in food of animal origin is relatively high, whereas the carnitine concentration in plant tissue is relatively low (11).

The current study was undertaken to characterize the transport of carnitine in *L. monocytogenes* cells and to establish whether carnitine is accumulated or converted into other compounds after uptake that serve as the actual osmoprotectants. Transport systems for the osmoprotectant betaine in several gram-negative and gram-positive bacteria (3, 12, 17, 20, 32, 37, 42, 45), including *L. monocytogenes* (29, 36), have been described and have been most extensively studied in *Escherichia coli* and *Salmonella typhimurium* (12). Uptake of carnitine has been observed in *Pseudomonas aeruginosa*. This species can use carnitine as the sole source of carbon and nitrogen (27). Members of the family *Enterobacteriaceae* do not assimilate the carbon and nitrogen skeleton of L-carnitine but are able to metabolize carnitine during anaerobic growth, via crotonobetaine which serves as an external electron acceptor, to τ-butyrobetaine in the presence of other substrates which act as carbon and/or nitrogen sources. Recently, Eichler and coworkers (14) characterized the *cai* genes of *E. coli*, which encode the carnitine pathway. The genes belong to the *caiTABCDE* operon, which is only transcribed during anaerobic growth in the presence of carnitine. It was suggested that CaiT is the transport system for carnitine in *E. coli*. CaiT counts 12 or 14 putative

hydrophobic membrane-spanning regions being indicative for a secondary transporter (14, 40).

In this study the presence of an ATP-dependent, high affinity L-carnitine transport system in *L. monocytogenes* is demonstrated, which is distinct from the previously described betaine transport system in this organism (29, 36). Furthermore, it is shown that accumulated L-carnitine is not metabolized in *L. monocytogenes,* which presents evidence for its role as an osmoprotectant. Possible roles of the L-carnitine transporter in the cold tolerance of *L. monocytogenes* and in the intracytoplasmic growth of this food-borne human pathogen in mammalian cells are discussed.

Materials and Methods

Bacterial strain and media. *L. monocytogenes* Scott A was grown in complex medium, brain heart infusion (BHI), or in the defined minimal medium (DM) of Premaratne *et al.* (44). This medium contains a defined mixture of amino acids and vitamins with glucose as the major carbon source. Media were supplemented with NaCl, KCl, or sucrose to raise the osmotic strength as required.

Transport assays. Cells were inoculated into 100 ml of medium in 300-ml Erlenmeyer flasks as a 1/20 dilution from an overnight culture in identical medium, grown at 30°C with agitation (150 rpm) in a shaker-incubator (Gallenkamp, Griffin Europe, Breda, The Netherlands) to mid-exponential phase, and harvested by centrifugation. Cells were subsequently washed twice in 50 mM potassium phosphate (pH 6.9)-5 mM $MgSO_4$ containing 50 μg of chloramphenicol per ml and stored on ice until use. In experiments in which $HgCl_2$, vanadate, arsenate, *N,N'*-dicyclohexylcarbodiimide (DCCD), or diethylstilbestrol (DES) were used, the potassium phosphate was replaced by 50 mM potassium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5).

Transport assays were conducted at 30°C in the buffers described above unless indicated otherwise. Cells were incubated (at an optical density at 600 nm (OD_{600}) of approximately 1) in the presence of 0.5% (wt/vol) glucose prior to the addition of L-[^{14}C]carnitine. Samples of 100 μl were withdrawn, and uptake was stopped by addition of 2 ml of cold buffer and immediately filtered over 0.2-μm-pore-size cellulose nitrate filters (Schleicher and Schuell GmbH, Dassell, Germany) under vacuum, and the filters were washed with 2 ml of cold buffer. The filters were inserted into plastic scintillation vials with 4 ml of scintillant, and the radioactivity was subsequently measured with a liquid scintillation counter (model 1600TR; Packard Instruments Co., Downers Grove, Ill.).

Fate of intracellular L-[^{14}C]carnitine. Cells grown in DM at 30°C with or without 0.5 M NaCl were harvested at an OD_{600} of 0.6, washed twice, and resuspended (OD_{600}, 20) in 50 mM potassium phosphate (pH 6.9) with 5 mM $MgSO_4$ and 50 μg of chloramphenicol per ml and stored on ice. After preincubation of cells (OD_{600}, 1.0) for 3 min, uptake was started by addition of L-[N-*methyl*-^{14}C]carnitine (final concentration, 24 μM). After 15 min, 300-μl samples were taken and the cells were separated from the external medium by centrifugation (10 min, 8,000 x *g*). The supernatant was stored on ice, and the cell pellets were washed twice with 50 mM potassium phosphate (pH 6.9) containing 5 mM $MgSO_4$ and 50 μg of chloramphenicol per ml. Radioactivity was extracted subsequently with 100 μl of 5% (vol/vol) perchloric acid and 10 mM EDTA on ice for 45 min. The extracts were neutralized with an equal amount of a solution of 1 M KOH and 1 M $KHCO_3$. Aliquots of 10 μl of perchloric acid extracts and supernatant were chromatographed on Silica Gel G (Merck AG, Darmstadt, Germany) thin-layer plates by using a methanol-ammonia (75:25) solvent system (15). Radioautographs were made with Kodak X-ray films.

Metabolism of L-[^{14}C]carnitine by *S. cerevisiae* was examined in the presence of acetyl coenzyme (acetyl-CoA). Acetyl-CoA is supposed to be involved in the conversion of carnitine during lipid metabolism in eukaryotic cells (8). For this purpose, cell extract (CE) of *S. cerevisiae* was incubated with L-[^{14}C]carnitine (24 μM) in the presence of 250 μM acetyl-CoA for 15 minutes. For comparison, CE of *L. monocytoges* Scott A grown in DM was obtained by sonification as described (49) and subsequently incubated as was CE of *S. cerevisiae*. Aliquots (7 μl) of these incubations were spotted on Silica Gel G and chromatographed and analyzed as described above.

Measurement of the membrane potential and intracellular ATP concentration. The transmembrane electrical potential ($\Delta\psi$) was determined with an electrode specific for the lipophilic cation tetraphenylphosphonium (final concentration, 4 μM), as described previously (46). Cells of *L. monocytogenes* were prepared for measurements as described above and incubated (OD_{600}, 0.8) at 30°C in 50 mM potassium phosphate (pH 6.9) in the presence of 0.5 % (wt/vol) glucose, unless indicated otherwise. By adding the potassium proton exchanger nigericin (2 μM), the pH gradient (alkaline inside) was dissipated such that the proton motive force (PMF) was composed of the $\Delta\psi$ only. The intracellular ATP concentration was determined as described previously (1). A cytoplasmic volume of 3 μl per mg of cell protein was used (35). In experiments in which DCCD, DES, $HgCl_2$, and the phosphate analogs arsenate and vanadate were used, the potassium phosphate was replaced by 50 mM potassium HEPES (pH 7.5).

Protein determination. Protein concentrations were determined by the method of Lowry *et al.* (31) with bovine serum albumin as a standard.

Chemicals. L-[N-*methyl*-^{14}C]carnitine (1.96 TBq/mol) was obtained from DuPont. BHI was from Difco Laboratories, Detroit, Mich. Other chemicals were reagent grade and obtained from Sigma Chemical Company, St. Louis, Mo., or other commercial sources.

Results

Transport of L-carnitine in *L. monocytogenes*. Uptake of L-[N-*methyl*-^{14}C]carnitine (final concentration, 19 μM) was examined in cells of *L. monocytogenes* grown in BHI. In the absence of an energy source, a low rate of L-carnitine transport was detected whereas a strong stimulation of the uptake was observed in the presence of glucose (Fig. 1A). When the cells were permeabilized with chloroform (1% (vol/vol)), L-carnitine uptake was completely abolished (Fig. 1A). These results suggest that the uptake of L-carnitine is an energy-dependent process. The potassium proton exchanger nigericin (2 μM), which dissipated the transmembrane pH gradient, had no severe effect on L-carnitine transport (Fig. 1B). With the potassium ionophore valinomycin (1.5 μM), which collapsed the membrane potential $\Delta\psi$, L-carnitine transport was only partially inhibited (approximately 50% of the activity of the control). Addition of both valinomycin (1.5 μM) and nigericin (2 μM) resulted in a complete dissipation of the PMF (not shown), whereas uptake of L-carnitine was still functioning (Fig. 1B). The transport of L-[^{14}C]lysine (unpublished observation) and prolyl-[^{14}C]alanine, which are driven by the PMF, was completely abolished in the presence of both valinomycin (1 μM) and nigericin (1 μM) (49). These experiments suggest that L-carnitine transport can proceed in the absence of a PMF.

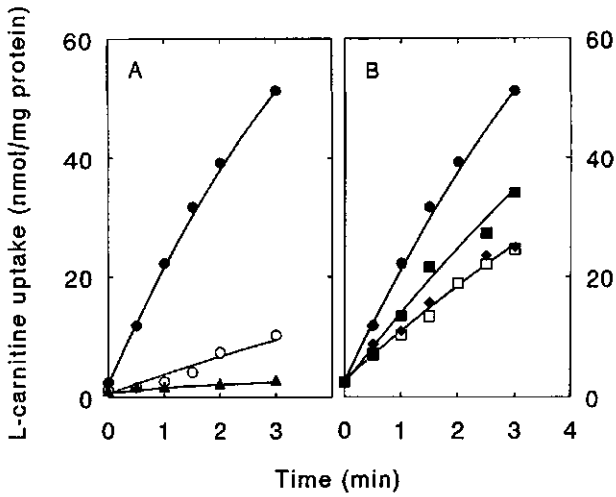

Figure 1. Transport of L-carnitine in *L. monocytogenes* Scott A. Uptake of L-[N-*methyl*-^{14}C]carnitine (final concentration, 19 μM) was performed in BHI-grown cells at 30°C in 50 mM potassium phosphate (pH 6.9) containing 5 mM $MgSO_4$ and 50 μg of chloramphenicol per ml. Transport was assayed after preincubation for 3 min. (A) Uptake in the absence (○) and presence (●) of 0.5% (wt/vol) glucose and by cells treated with 1% (vol/vol) chloroform (▲). (B) Effect of nigericin (2 μM) (■), valinomycin (1.5 μM) (□), or nigericin plus valinomycin (◆) on glucose-energized L-[^{14}C]carnitine transport (●).

Fate of translocated L-carnitine. The ability of *L. monocytogenes* to metabolize L-carnitine was investigated by chromatographic analysis of radioactivity accumulated from L-[^{14}C]carnitine uptake in *L. monocytogenes* cells grown in DM. Only a single ^{14}C-labeled compound was detected which moved to the same position as L-[^{14}C]carnitine (Fig. 2, lanes 1 and 2). This indicates that intracellular L-carnitine accumulates as such in the cytoplasm and is not catabolized. This is consistent with the observation that carnitine cannot serve as a carbon or nitrogen source for *L. monocytogenes* (48). Almost all L-[^{14}C]carnitine was taken up by *L. monocytogenes* cells since only very small amounts of radioactivity could be detected in the supernatant (Fig. 2, lane 3). Results with cells grown in DM with 0.5 M NaCl (Fig. 2, lanes 4 and 5) were indistinguishable from those obtained in the absence of NaCl. Furthermore, L-carnitine metabolism was not observed in CE of *L. monocytogenes* incubated with acetyl-CoA, whereas with CE of *S. cerevisiae* a significant metabolism of L-[^{14}C]carnitine was observed (Fig. 2, lanes 6 and 7). In eukaryotic cells, metabolism involves a reaction in which carnitine is converted into acylcarnitine with the intervention of acetyl-CoA (8). From these results it can be concluded that uptake of carnitine by *L. monocytogenes* in the absence of a PMF (Fig. 1B) is not due to rapid intracellular metabolism.

Energetics of the L-carnitine transport system. The energetics of L-carnitine transport

were characterized in detail by analysis of the effects of the phosphate analogs arsenate and vanadate, the H^+-ATPase inhibitor DCCD, and DES on bioenergetic parameters and L-carnitine uptake in *L. monocytogenes* (Table 1). The addition of DCCD resulted in a complete dissipation of the PMF, whereas the intracellular ATP concentration was increased (127% of control). L-carnitine transport is stimulated under these conditions (146% of control). In the presence of DES, a slight stimulation of the PMF, an increase in the intracellular ATP concentration (133% of control), and an increase in L-carnitine uptake (170% of control) were observed. In the presence of arsenate and vanadate the magnitude of the PMF and the intracellular ATP concentrations were slightly decreased. Transport of L-carnitine was significantly inhibited under these conditions. Combining these results, it can be concluded that ATP or an equivalent energy-rich phosphorylated intermediate supplies the energy for the carnitine translocation process.

The addition of both nigericin (2 μM) and valinomycin (1.5 μM) to *L. monocytogenes* cells which had been preloaded with L-[^{14}C]carnitine (19 μM) did not result in efflux of radioactive label from the cells. Moreover, *L. monocytogenes* did not displace intracellular L-[^{14}C]carnitine for unlabeled L-carnitine when a 40-fold excess of L-carnitine was added to cells preloaded with L-[^{14}C]carnitine (data not shown). These results give evidence for a kinetically irreversible L-carnitine uptake system, which is also characteristic of ATP-dependent transport systems (38).

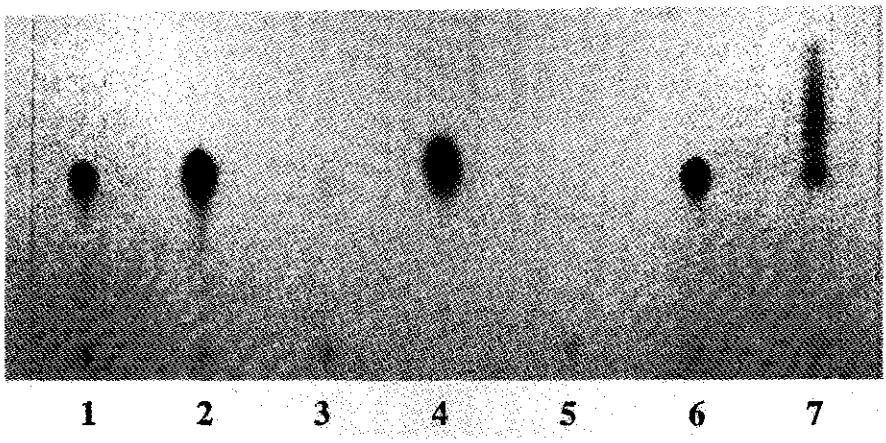

Figure 2. Fate of intracellular L-carnitine in *L. monocytogenes* Scott A. Uptake of L-[N-*methyl*-^{14}C]carnitine (final concentration, 24 μM), extraction from the cells, and chromatographic analysis were performed as described in Materials and Methods. Lane 1, L-[N-*methyl*-^{14}C]carnitine. Lanes 2 and 3, perchloric acid CE (3 ng of protein) and supernatant of cells grown in DM, respectively. Lanes 4 and 5, perchloric acid CE (3 ng of protein) and supernatant of cells grown in DM with 0.5 M NaCl, respectively. Lanes 6 and 7, sonicated CE of *L. monocytogenes* (2 ng of protein) and *S. cerevisiae* (0.8 ng of protein), respectively, incubated in the presence of acetyl-CoA (250 μM).

Table 1. Effect of DCCD, arsenate and vanadate on bioenergetic parameters and L-[^{14}C]carnitine uptake in *Listeria monocytogenes*[a].

ATPase inhibitor or phosphate analog added[b]	PMF (mV)[c]	ATP_{in} (mM)[c]	L-[^{14}C]carnitine uptake (nmol min^{-1} mg of $protein^{-1}$)[c]
None	-128 (100)[d]	6.5 (100)	7.6 (100)
DCCD (0.2 mM)	0 (0)	8.3 (127)	11.1 (146)
DES (0.15 mM)	-130 (108)	8.8 (133)	12.9 (170)
Arsenate (0.5 mM)	-119 (93)	5.0 (76)	1.0 (13)
Vanadate (0.5 mM)	-118 (93)	5.6 (85)	1.1 (14)

[a]Uptake of L-[^{14}C]carnitine (final concentration, 19 μM) was determined in BHI-grown cells incubated at 30°C in 50 mM potassium HEPES (pH 7.5) containing 0.5% (wt/vol) glucose, 5 mM $MgSO_4$ and 50 μg of chloramphenicol per ml. The potassium-proton exchanger nigericin was added (final concentration, 2 μM) to dissipate the pH gradient (ΔpH) such that the PMF was composed of the membrane potential only (PMF = $\Delta\psi$). Uptake was started after preincubation for 5 min by addition of L-[^{14}C]carnitine. The $\Delta\psi$ was determined in parallel experiments, and samples were taken for determination of intracellular ATP concentrations. The rate of uptake of L-[^{14}C]carnitine was determined between 5 and 10 min, since the effect of the phosphate analogs arsenate and vanadate became evident after about 5 min.

[b]DCCD, DES, arsenate, and vanadate were added to the indicated final concentrations, after preincubation for 5 min. After incubation for another 5 min, uptake was started by the addition of L-[^{14}C]carnitine.

[c] The values for PMF, intracellular ATP concentration (ATP_{in}), and initial rate of uptake of L-[^{14}C]carnitine in the control experiment (no addition) were set at 100%. Relative percentages in the presence of inhibitors are given in parentheses.

Feedback regulation of L-carnitine transport. L-[^{14}C]carnitine was taken up at a high rate in cells grown in DM (Fig. 3), reflecting that carnitine uptake is constitutively expressed rather than induced during growth in the carnitine-containing BHI (29). Uptake rates in DM-grown cells, however, appeared to be four- to fivefold higher than those in BHI-grown cells. To investigate whether the observed lower L-[^{14}C]carnitine uptake rates in BHI-grown cells are caused by the presence of pre-accumulated unlabeled carnitine in the cell, L-[^{14}C]carnitine transport was studied in *L. monocytogenes* cells grown in DM in the presence of different L-carnitine concentrations. Growth in DM containing 0.02 mM L-carnitine resulted in a markedly lower rate of L-carnitine transport in these cells than in cells grown in DM (control). Growth in DM in the presence of higher L-carnitine concentrations (0.2 mM and 2 mM) had even a more pronounced effect on the rate of L-carnitine transport (Fig. 3). To exclude a possible repression of the synthesis of the transporter by L-carnitine, the following experiment was devised. *L. monocytogenes* was grown in DM, harvested at mid-exponential phase, and washed twice in 50 mM potassium phosphate (pH 6.9)-5 mM $MgSO_4$ containing 50 μg of chloramphenicol per ml. Cells were subsequently incubated for 45 min with

different L-carnitine concentrations in the presence of chloramphenicol, which inhibits *de novo* protein synthesis. The cells were washed three times and finally subjected to L-[^{14}C]carnitine as described in Materials and Methods. Relative uptake rates were comparable (not shown) to those shown in Fig. 3. These data indicate that the L-carnitine transport system is feedback inhibited by intracellular L-carnitine.

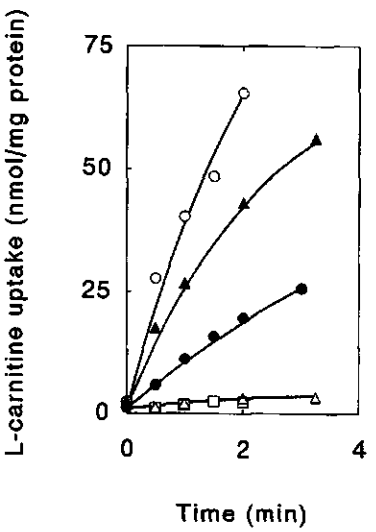

Figure 3. Feedback regulation of L-carnitine transport in *L. monocytogenes* Scott A. The influence of the presence of L-carnitine during growth was determined. Transport assays were performed in 50 mM potassium phosphate (pH 6.9) containing 5 mM $MgSO_4$ and 50 μg of chloramphenicol per ml. Uptake was started after preincubation with 0.5% (wt/vol) glucose by the addition of L-[^{14}C]carnitine (final concentration, 19 μM). ●, cells grown in BHI; ○, cells grown in DM; ▲, cells grown in DM plus 0.02 mM L-carnitine; Δ, cells grown in DM plus 0.2 mM L-carnitine; □, cells grown in DM plus 2 mM L-carnitine.

Kinetics of L-carnitine transport. Initial rates of transport were determined over a wide range of L-carnitine concentrations (1 to 500 μM) with cells grown in DM. An Eadie-Hofstee plot of the data was monophasic, suggesting the presence of a single carnitine transport system, and revealed a K_m value of 10 μM and a V_{max} value of 48 nmol of carnitine transported per min per mg of cell protein (data not shown). The same K_m value was found for BHI-grown *L. monocytogenes*, indicating that no additional L-carnitine uptake system is induced during growth in this carnitine-containing medium. A V_{max} value of 18 nmol min^{-1} mg of $protein^{-1}$ was obtained in BHI-grown cells. The lower V_{max} in BHI-grown cells can be explained by feedback regulation of the transport system (see above).

pH dependence of L-carnitine transport. The effect of the external pH (pH_{ext}) on the rate of L-[^{14}C]carnitine uptake was studied in the absence and presence of the potassium

Table 1. Effect of DCCD, arsenate and vanadate on bioenergetic parameters and L-[^{14}C]carnitine uptake in *Listeria monocytogenes*[a].

ATPase inhibitor or phosphate analog added[b]	PMF (mV)[c]	ATP_{in} (mM)[c]	L-[^{14}C]carnitine uptake (nmol min^{-1} mg of $protein^{-1}$)[c]
None	-128 (100)[d]	6.5 (100)	7.6 (100)
DCCD (0.2 mM)	0 (0)	8.3 (127)	11.1 (146)
DES (0.15 mM)	-130 (108)	8.8 (133)	12.9 (170)
Arsenate (0.5 mM)	-119 (93)	5.0 (76)	1.0 (13)
Vanadate (0.5 mM)	-118 (93)	5.6 (85)	1.1 (14)

[a]Uptake of L-[^{14}C]carnitine (final concentration, 19 μM) was determined in BHI-grown cells incubated at 30°C in 50 mM potassium HEPES (pH 7.5) containing 0.5% (wt/vol) glucose, 5 mM $MgSO_4$ and 50 μg of chloramphenicol per ml. The potassium-proton exchanger nigericin was added (final concentration, 2 μM) to dissipate the pH gradient (ΔpH) such that the PMF was composed of the membrane potential only (PMF = $\Delta\psi$). Uptake was started after preincubation for 5 min by addition of L-[^{14}C]carnitine. The $\Delta\psi$ was determined in parallel experiments, and samples were taken for determination of intracellular ATP concentrations. The rate of uptake of L-[^{14}C]carnitine was determined between 5 and 10 min, since the effect of the phosphate analogs arsenate and vanadate became evident after about 5 min.

[b]DCCD, DES, arsenate, and vanadate were added to the indicated final concentrations, after preincubation for 5 min. After incubation for another 5 min, uptake was started by the addition of L-[^{14}C]carnitine.

[c] The values for PMF, intracellular ATP concentration (ATP_{in}), and initial rate of uptake of L-[^{14}C]carnitine in the control experiment (no addition) were set at 100%. Relative percentages in the presence of inhibitors are given in parentheses.

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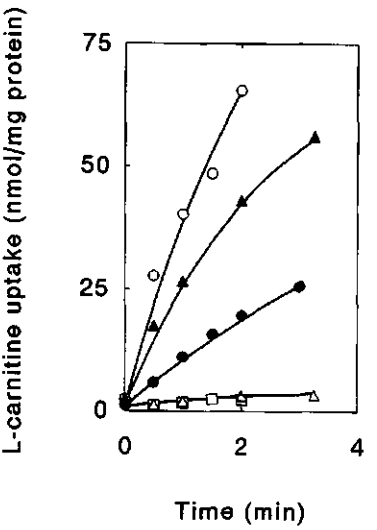

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pH dependence of L-carnitine transport. The effect of the external pH (pH_{ext}) on the rate of L-[^{14}C]carnitine uptake was studied in the absence and presence of the potassium proton exchanger nigericin (2 μM). Without nigericin, a decrease in medium pH led to a

decrease in the initial rate of uptake, which was accompanied by a decrease in the intracellular ATP concentrations (Fig. 4). Over the pH_{ext} range from 5.5 to 8, *L. monocytogenes* Scott A maintains its internal pH (pH_{in}) relatively constant; the pH_{in} increases from 7.2 at pH_{ext} 5.5 to 8.1 at pH_{ext} 8 (9). The addition of nigericin, which dissipates the pH gradient across the cytoplasmic membrane, did not affect intracellular ATP levels (Fig. 4A). Transport of L-carnitine was completely inhibited in the presence of nigericin at acidic pH values (Fig. 4B). Apparently, transport of L-carnitine is dependent on the intracellular pH with optimum activity at alkaline pH values.

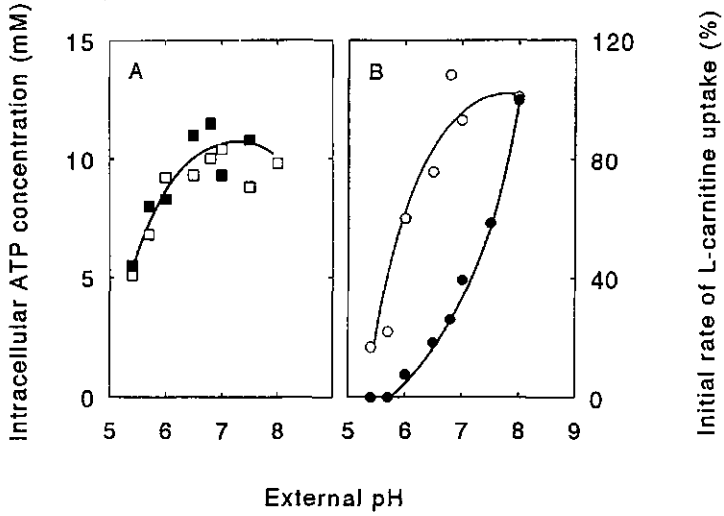

Figure 4. Effect of nigericin on ATP levels and L-carnitine transport in *L. monocytogenes* Scott A at different external pH values. (A) Intracellular ATP concentrations were measured in BHI-grown cells in 50 mM potassium phosphate (pH 6.9) containing 0.5% glucose (wt/vol), 5 mM $MgSO_4$, and 50 μg of chloramphenicol per ml at different external pH values in the absence (□) and presence (■) of 2 μM nigericin. (B) Initial uptake rates of L-[^{14}C]carnitine (final concentration, 19 μM) were determined over the first 2 min in parallel experiments with these cells in the absence (○) or presence (●) of 2 μM nigericin at the indicated pH values. In the presence of nigericin the internal pH was equal to the external pH. Cells were preincubated for 3 min before the addition of L-[^{14}C]carnitine.

Osmotic effects. Osmoprotection by L-carnitine at different L-carnitine concentrations was determined. A concentration as low as 10 μM appeared to be sufficient to stimulate growth of *L. monocytogenes* in high osmolarity DM (data not shown). This suggests that the high-affinity L-carnitine transporter can play an important role in the adaptation of *L. monocytogenes* to growth at high osmolarity. Initial rates of L-carnitine (19 μM) uptake in *L. monocytogenes* cells grown in BHI containing 0.5 M NaCl were found to be indistinguishable from those in cells grown in the absence of NaCl (data not shown). Furthermore, the initial uptake rate of L-carnitine (19 μM) was demonstrated to be similar in assay buffers in which the osmolarity was increased by addition of 0.4 M NaCl, 0.4 M KCl, or 0.6 M sucrose (not

shown). The activity of the L-carnitine transporter is consequently not stimulated by a rise in the extracellular osmolarity.

Temperature dependence of L-carnitine transport. Considering the ability of *L. monocytogenes* to grow at refrigeration temperature, it was of interest to determine the temperature dependence of L-carnitine uptake. The initial rate of L-carnitine transport of *L. monocytogenes* cells grown at 30°C decreased with decreasing assay temperature (Fig. 5). The uptake of L-carnitine at 30°C was approximately 30 nmol min^{-1} mg of $protein^{-1}$ and was decreased to 3 nmol min^{-1} mg of $protein^{-1}$ at 5°C. It should be stressed that the uptake of L-carnitine at 5°C is still significant. Strikingly, cells grown at 7°C transported L-carnitine at a rate of approximately 3 nmol min^{-1} mg of $protein^{-1}$ over the whole temperature range assayed (Fig. 5).

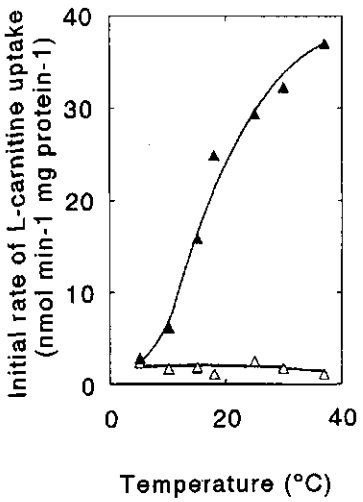

Figure 5. Effect of temperature on L-carnitine transport in *L. monocytogenes* Scott A. Initial uptake rates of L-[^{14}C]carnitine were determined at different temperatures in cells grown at 30°C (▲) and 7°C (Δ). Transport assays were performed in 50 mM potassium phosphate (pH 6.9) containing 5 mM $MgSO_4$ and 50 μg of chloramphenicol per ml. Uptake was started after preincubation with 0.5% (wt/vol) glucose by the addition of L-[^{14}C]carnitine (final concentration, 19 μM).

Specificity of the L-carnitine transport system. The specificity of the carnitine transport system was investigated by studying the initial rates of L-[^{14}C]carnitine uptake in assay mixtures in which structural analogs were introduced at a 10- and 100-fold excess of L-carnitine (19 μM, final concentration). The carnitine transport system was found to be highly specific for L-carnitine, since D-carnitine was not an efficient inhibitor (Table 2). L-proline and betaine displayed even at a 100-fold excess only a weak inhibitory effect. Of the other analogs investigated, only butyrobetaine and acetylcarnitine competed efficiently with L-carnitine for uptake.

Table. 2. Uptake of L-[^{14}C]carnitine by *L. monocytogenes* in the presence of excess unlabeled analog

Analog	Uptake of L-[^{14}C]carnitine (% activity)[a]	
	10-fold unlabeled analog	100-fold unlabeled peptide
Choline	100	86
Betaine	100	70
γ-Aminobutyric acid	100	100
Acetylcholine	102	100
Pivalic acid	102	92
L-Proline	100	80
D-Carnitine	51	33
DL-Carnitine	17	0
L-Carnitine	8	0
Acetylcarnitine	15	0
Octanoylcarnitine	100	55
Butyrobetaine	13	0

[a] Uptake of L-[^{14}C]carnitine (final concentration, 19 μM) was determined in the presence of 10- and 100-fold excess of simultaneously added unlabeled analog. The initial rate of uptake was determined over the first 2 min in BHI-grown cells after preincubation for 3 min in 50 mM potassium phosphate (pH 6.9) containing 0.5% (wt/vol) glucose, 5 mM $MgSO_4$, and 50 μg of chloramphenicol per ml. The initial rate of L-[^{14}C]carnitine uptake (final concentration, 19 μM) at 30°C (100% activity) was 18 nmol min^{-1} mg of $protein^{-1}$. Data represent the mean of at least two experiments. Absolute deviations in the data were less than 10%.

Effect of sulfhydryl group (SH) reagents on the L-carnitine transport system. The effect of various SH-modifying reagents on the activity of the L-carnitine transporter was examined in the presence of nigericin (2 μM). L-carnitine uptake was not affected by mersalyl at a concentration of 200 μM. *N*-ethylmaleimide (NEM) and *p*-chloromercuribenzene sulfonate (*p*CMBS) inhibited L-carnitine uptake to a certain extent at the concentrations indicated, whereas $HgCl_2$ blocked transport completely (Fig. 6). Parallel to the transport assays, $\Delta\psi$ was determined and samples were taken for determination of intracellular ATP concentrations. In control cells the $\Delta\psi$ was -130 mV and the intracellular ATP concentration was approximately 6.8 mM. The presence of NEM, *p*CMBS, or mersalyl did not influence the magnitude of the PMF (data not shown). The intracellular ATP concentration was not altered by NEM, whereas with *p*CMBS or mersalyl the intracellular ATP concentrations increased (210% and 300% of control, respectively, data not shown). The addition of $HgCl_2$ resulted in a complete dissipation of the PMF and the intracellular ATP

concentration decreased drastically (15% of control) (data not shown). These results indicate that inactivation of the L-carnitine transport system by NEM and *p*CMBS is due to specific reactions with SH-groups of the transporter.

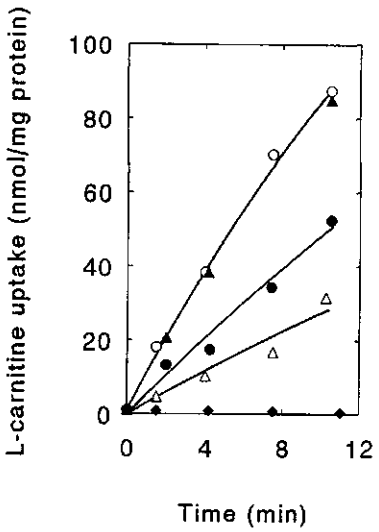

Figure 6. Effect of SH reagents on the uptake of L-carnitine by *L. monocytogenes* Scott A. Cells were incubated in 50 mM potassium phosphate (pH 6.9) containing 2 μM nigericin, 5 mM $MgSO_4$ and 50 μg of chloramphenicol per ml. Experiments with $HgCl_2$ were performed in 50 mM potassium HEPES (pH 7.5). After 5 min of preincubation, SH reagents were added, and after incubation for another 5 min, uptake was started by the addition of L-[^{14}C]carnitine (final concentration, 19 μM). Symbols: ▲, no additions; ●, NEM (500 μM); Δ, *p*CMBS (100 μM); ○, *O*-[3-hydroxymercuri-2-methoxypropyl]carbamylphenoxyacetate (mersalyl) (200 μM) and ◆, $HgCl_2$ (50 μM).

Discussion

Carnitine has been shown to stimulate growth of *L. monocytogenes* at high osmotic strength (7). The results presented here demonstrate that accumulation of L-carnitine is mediated via a specific transport system with a high affinity and a high capacity for L-carnitine (K_m = 10 μM; V_{max} = 48 nmol min^{-1} mg of protein^{-1}). L-carnitine was detected in an unmodified form inside the cells (Fig. 2), which is in agreement with the observation that it cannot serve as a carbon or nitrogen source for *L. monocytogenes* (48). These observations unequivocally show that L-carnitine is metabolically inert in *L. monocytogenes* and that the L-carnitine transporter has a crucial role in the supply of this bacterium with L-carnitine.

The accumulation of L-carnitine in *L. monocytogenes* was found to require metabolic energy since the omission of glucose abolished uptake. It was deduced that the driving force for uptake is supplied by ATP or another energy-rich phosphorylated intermediate, on the basis of the following properties of the system. *(i)* The uptake of L-carnitine was only partially inhibited in the presence of nigericin, an electroneutral K^+/H^+ ionophore, and

valinomycin, a K^+- specific ionophore (Fig. 1B), indicating that transport can proceed in the absence of an electrochemical gradient for protons. Extra evidence for the exclusion of the involvement of the PMF came from the observation that in the presence of the ATPase inhibitor DCCD, the PMF was completely abolished, whereas the uptake of L-carnitine was stimulated twofold, concomitant with a rise in the intracellular ATP concentration (Table 1). DCCD most likely blocks conversion of the glycolytically generated ATP into a PMF, owing to the absence of a proton-translocating electron transport system. *(ii)* Exchange of intracellular L-[^{14}C]carnitine against a high concentration of extracellular nonlabeled L-carnitine did not occur. Efflux of L-[^{14}C]carnitine upon addition of the ionophores valinomycin and nigericin was also not observed (data not shown). *(iii)* The L-carnitine accumulation ratio (in/out) can reach a value of about 10^5, which exceeds the thermodynamic limits set by the PMF for secondary transport systems (40). *(iv)* L-carnitine transport was susceptible to arsenate and vanadate, which interact with reactions involving high-energy phosphate bonds, whereas these compounds had a small effect on the PMF and caused only a slight decrease in the amount of intracellular ATP (Table 1). These findings indicate that inhibition of L-carnitine transport by arsenate and vanadate might be due to inhibition of the transport system itself. The L-carnitine transport system most likely belongs to the bacterial ATP-driven binding-protein-dependent transport systems of the superfamily of ABC transporters which are widely distributed among all living organisms (4, 18). Many of these ATP-driven transport systems are sensitive to vanadate (30, 41). In general, an ABC transporter comprises two transmembrane and two cytoplasmic domains. Bacteria possess an extra binding subunit for the import of substrates, which is periplasmic in gram-negative bacteria, whereas in gram-positive bacteria it is a lipoprotein anchored to the cytoplasmic membrane (18). Recent genetic studies revealed that transport of arginine in *L. monocytogenes* is also mediated by an ABC transporter, which synthesis is induced during intracellular growth in infected mammalian cells (26).

Variations in the magnitude of the ΔpH influenced the activity of the transport (Fig. 4B) despite the dependence of the L-carnitine transport on phosphate bond energy. *L. monocytogenes* Scott A maintains its pH_{in} relatively constant over a pH_{ext} range of 5 to 8 (9, 10). These results might indicate that the pH_{ext} alters the affinity of the transport system by affecting the available concentration of transported solute via protonation and deprotonation or that interactions of protons with the transporter alter its catalytic activity. The apparent relationship between L-carnitine transport and pH_{ext}, however, can also be attributed to the decline in intracellular ATP concentration with decreasing pH (Fig. 4B). The dependence of the initial rate of L-carnitine transport on the intracellular pH showed that L-carnitine transport requires a neutral or alkaline cytoplasm for optimum activity. Regulatory intracellular pH effects on ATP-dependent transport systems can be classified as allosteric rather than catalytic since protons are not directly involved in the energy coupling mechanism. ATP-dependent transport systems, including the L-carnitine transport system described here, apparently all function optimally at slightly alkaline pH values (2, 24, 39).

The different L-carnitine uptake rates obtained in cells grown in BHI and DM can be explained by feedback regulation of the transport system by intracellular L-carnitine (Fig. 3).

Feedback (*trans*) inhibition acts as a regulatory device to prevent solute accumulation to unacceptable high intracellular levels. This type of regulation of transport is typical for ATP-dependent transport systems, since these systems, like the L-carnitine transporter in *L. monocytogenes*, are unidirectional and can catalyze the uptake of solutes to high accumulation levels. Regulation of the activity of a compatible solute transport system has not been described until recently. Pourkomailian and Booth (43) and Stimeling *et al.* (47) established that betaine transport in *Staphylococcus aureus* is subject to feedback inhibition by preaccumulated betaine. Similarly, *L. monocytogenes* closes down its L-carnitine uptake system once sufficient L-carnitine has been accumulated. In addition, we observed that the presence of betaine in the cultivation medium, which was shown not to be a substrate for the L-carnitine transporter (Table 2), also reduced the activity of the L-carnitine uptake system (not shown). The L-carnitine transporter might possibly be capable of sensing overall intracellular osmolality. Further detailed studies should lead to an understanding of the mechanisms by which the activity of the L-carnitine transport system is regulated.

Uptake studies with structural analogs of L-carnitine (Table 2) revealed that the transport system exhibits a high degree of substrate specificity. The system prefers the naturally occuring L-carnitine to its enantiomer D-carnitine. L-proline, which has been shown to confer osmotic tolerance in *L. monocytogenes* (7), was not recognized by the L-carnitine permease. Other amino acids were also not able to compete with L-carnitine uptake (data not shown) suggesting that L-carnitine transport is not mediated by any amino acid permease. Betaine did not compete for the uptake of L-carnitine, indicating that the L-carnitine transporter in *L. monocytogenes* differs from the recently described betaine transport system in this organism (29, 36). The finding that butyrobetaine, a carnitine precursor, hindered L-carnitine uptake while betaine was not inhibitory might indicate that the length of the alkylchain between the *N*-trimethyl group on the one end and the carboxyl group on the other end is crucial in recognition. Furthermore, the substrate specificity of the L-carnitine transporter of *L. monocytogenes* is distinct from the system that transports L-carnitine in *E. coli*, which is induced during anaerobic growth in the presence of carnitine. D-carnitine, betaine, butyrobetaine, and crotonobetaine were shown to be potent competitiors for carnitine transport in *E. coli* (14, 25). Surprisingly, L-carnitine transport in *L. monocytogenes* was strongly inhibited by a 10-fold excess of acetylcarnitine. The carnitine carrier in eukaryotic cells also mediates translocation of L-carnitine and acetylcarnitine in addition to other acylcarnitines (21, 33). The substrate specificity of the L-carnitine transport system in *L. monocytogenes* therefore shows some resemblance to that of the eukaryotic carnitine:acylcarnitine antiporter. SH reagents were applied to investigate whether the substrate binding site of the L-carnitine permease of *L. monocytogenes* resembles that of the eukaryotic transporter. The L-carnitine permease of *L. monocytogenes* is inhibited by the SH reagent NEM but was not sensitive to mersalyl (Fig. 6), whereas the eukaryotic carnitine:acylcarnitine antiporter is inhibited by NEM and mersalyl (34). Thus, in both systems sulfhydryl groups are involved in transport, but the dissimilarity in the ability of these SH reagents to block the two L-carnitine transporters suggests that reactive SH groups, near or in the substrate binding sites of the two systems are oriented differently. The

interaction of the L-carnitine transporter in *L. monocytogenes* with the organomercurial *p*CMBS (Fig. 6) might however be advantageous in its purification for further characterization. Interaction of organomercurials with transport carriers was suggested as a feasible purification method for transport systems (13). In conclusion, the L-carnitine transporter in *L. monocytogenes* is distinct from any transport system previously described and therefore represents a novel transport system for the translocation of L-carnitine.

The study reported here shows that *L. monocytogenes* is in the possession of a constitutive high-affinity uptake system for L-carnitine that enables the bacterium to scavenge L-carnitine when it is available at trace levels in foods (11). It was found that the transport capacity of the L-carnitine permease was very high in the absence of salt and that this activity could not be stimulated upon imposition of an osmotic stress. In contrast, betaine transport in *L. monocytogenes* is highly stimulated in the presence of high concentrations of NaCl (29, 36). This implicates that carnitine accumulation in *L. monocytogenes* can occur even under conditions of low osmolarity. The physiological role of this feature of the L-carnitine transporter is intriguing. Recently, it was demonstrated that betaine combats both osmotic and chill stress in *L. monocytogenes* (29). It is conceivable that chill tolerance in *L. monocytogenes* can also be conferred by the accumulation of L-carnitine. It was shown that *L. monocytogenes* cells grown at 7°C are able to take up L-carnitine at a rate of about 3 nmol min^{-1} mg of $protein^{-1}$ and in addition it was observed that L-carnitine can be accumulated to high concentrations under these conditions (data not shown). The possible role of the L-carnitine transport system in adaptation of *L. monocytogenes* to growth at low temperature is being further investigated.

It can also be speculated that the L-carnitine transporter is a means for *L. monocytogenes* to maintain its turgor pressure in the absence of stress. Gram-positive bacteria generally maintain a higher turgor than gram-negative bacteria, arising from their higher cytoplasmic concentrations of solutes at equivalent osmotic pressures (19, 23, 35, 50). Thus, *L. monocytogenes* would benefit from the uptake of compatible solutes even in a low-osmolarity environment. Moreover, the L-carnitine transporter may facilitate the intracellular growth and survival of *L. monocytogenes* in mammalian cells, which is an essential component of the pathogenesis of this organism. Both, carnitine and acetylcarnitine are present in the cytosol at concentrations high above the K_m values of the L-carnitine transport system in *L. monocytogenes* for these compounds (21). Considering that (*i*) the replication of *L. monocytogenes* in mammalian cells occurs in the cytoplasm, (*ii*) the osmolarity of the cytoplasm is probably relatively high, and (*iii*) both carnitine and acetylcarnitine (unpublished data) are effective osmoprotectants in *L. monocytogenes*, the availability of these osmolytes might increase the capacity of this human pathogen to grow in the host cell.

Acknowledgements

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Netherlands) for assistance in autoradiographic analysis.

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6

Betaine and L-carnitine transport in response to osmotic signals in *Listeria monocytogenes* Scott A

Annette Verheul, Erwin Glaasker, Bert Poolman and Tjakko Abee

Abstract

The natural occurring compatible solutes betaine and L-carnitine allow the food-borne pathogen *Listeria monocytogenes* to adjust to environments of high osmotic strength. Betaine and L-carnitine are taken up by separate highly specific transport systems that are driven by the electrochemical ion gradient and ATP, respectively. The initial uptake rates of betaine and L-carnitine are not influenced by an osmotic upshock, but the duration of transport of both osmolytes is directly related to the osmotic strength of the medium. Regulation of both betaine and L-carnitine uptake is subject to *trans* inhibition by preaccumulated solute. Importantly, internal betaine not only inhibits the transport of external betaine but also that of L-carnitine and, similarly, internal L-carnitine inhibits both betaine and L-carnitine transport. The *trans* inhibition is alleviated upon osmotic upshock which suggests that alterations in membrane structure are transmitted to the binding pockets for betaine and L-carnitine of both transporters at the inner surface of the membrane. Upon osmotic downshock, betaine and L-carnitine are rapidly released by *L. monocytogenes* as a consequence of activation of a channel-like activity. The osmolyte sensing mechanism described is new and consistent with various unexplained observations of osmoregulation in other bacteria.

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Introduction

Foodborne listeriosis caused by *Listeria monocytogenes* has emerged as a topic of considerable public health concern over the past decade. The infection is encountered in neonates, eldery persons, pregnant women and the immunocompromised, and symptoms may include septis, meningitis, infection of the central nervous system, abortion and stillbirth with fatality rates of approximately 25%. The ubiquitous distribution of *L. monocytogenes* in the environment and its relative high tolerance to environmental stresses such as low temperature and high osmotic strength contribute to its status as a hazard in minimally processed ready-to-eat refrigerated products (6).

Cold and salt tolerance in *L. monocytogenes* can be invoked by betaine and L-carnitine. Betaine is present in high concentrations in foods originating from plants whereas foods of animal origin generally have a high carnitine content (2, 13, 19, 28). The osmoprotective capacity of betaine is well-known among prokaryotic organisms, whereas, so far, L-carnitine has only been recognized as an osmolyte in *L. monocytogenes*, *Lactobacillus plantarum* and *Escherichia coli* (2, 11, 13). The involvement of betaine and L-carnitine in cold tolerance of the psychrotroph *L. monocytogenes* has recently been reported (13, 23, 28). Uptake of L-carnitine in *L. monocytogenes* is mediated by a constitutively expressed transporter that is driven by ATP. Competition experiments revealed that the L-carnitine transporter has high affinity for carnitine, acetylcarnitine and τ-butyrobetaine, and shows neglible affinity for betaine and L-proline (28). Uptake of betaine in *L. monocytogenes* is demonstrated to be driven by the electrochemical ion gradient (8, 19).

The present study was initiated to obtain information on the regulation of betaine and L-carnitine accumulation in *L. monocytogenes* in response to changes in the osmolarity of the growth medium. Since bacterial cells not only need to carry out processes to adapt to osmotic upshift, but require in addition ways to excrete intracellular solutes in order to overcome the danger of bursting in case of an osmotic downshock, the release of betaine and L-carnitine by *L. monocytogenes* was studied as well. Stretch-activated channels have been implicated in the fast non-specific solute efflux after osmotic downshock in both Gram-negative and Gram-positive bacteria (1). However, there is increasing evidence for specific osmolyte efflux systems, independent of the uptake system in *E. coli*, *Salmonella typhimurium*, *Ectothiorhodospira halochloris* and *L. plantarum* (9, 14, 15, 22, 26).

Materials and Methods

Bacterial strain and growth conditions. *Listeria monocytogenes* Scott A was grown in a chemically defined minimal medium (DM) as described (21). The osmolarity of this medium, as measured by freezing-point depression with an Osmomat 030 (Gonotec, Berlin, Germany), was 0.4 osmol/kg. High-osmolarity growth conditions were obtained by addition of NaCl or KCl (0.3 M or 0.5 M) to DM. Media were supplemented with betaine or L-carnitine as indicated. Cultures were grown at 37°C with shaking (200 rpm) until the optical density at 660 nm (OD_{660}) reached 0.65 (late-exponential growth phase).

Determination of intracellular osmolyte content. Cells were harvested by centrifugation and

washed twice by resuspension and centrifugation in 50 mM potassium phosphate (pH 6.8)-5 mM $MgSO_4$, which is isotonic with the growth medium. The pelleted cells were freeze-dried and extracted by the procedure of Galinski and Herzog (7), which is a modification of the Bligh and Dyer (3) method. Betaine and L-carnitine concentrations were determined by refractive index after HPLC using a LiChrosphere 100-NH_2, 5 μm column (Merck, Darmstadt, Germany) at a flow rate of 1 ml/min at 45°C with a mobile phase of 80:20 (vol/vol) acetonitril-20 mM potassium phosphate (pH 7.0).

Transport studies. Cells at an OD_{660} of 20 in 50 mM potassium phosphate (pH 6.8) containing 5 mM $MgSO_4$, unless mentioned otherwise, were stored on ice until use. Cells (OD_{660} of 1) were preenergized at 30°C with 10 mM glucose for 5 min prior to the addition of radiolabelled substrate (final concentration of 1.3 mM). Potential competitors of betaine and L-carnitine transport were introduced together with the label at a 80-fold excess. The buffer osmolarity was raised by adding aliquots of 3 M KCl or 3 M NaCl or buffer simultaneously with the radiolabeled substrate. Samples were withdrawn and uptake was stopped by addition of 2 ml of cold LiCl (0.1 to 1 M, depending on the osmolarity of the assay buffer) and the cells were collected on 0.2-μm-pore-size cellulose nitrate filters (Schleicher and Schuell GmbH, Dassell, Germany) under vacuum; the filters were washed with another 2 ml of LiCl and the radioactivity trapped in the cells was measured with a liquid scintillation counter (model 1600TR; Packard Instruments Co., Downers Grove, Ill.). Uptake of osmolytes was normalized to total cellular protein, which was determined by the method of Lowry *et al.* (17) using bovine serum albumin as a standard. A specific cytoplasmic volume of 3 μl per mg of cell protein was used for the calculation of the internal substrate concentration (18).

Osmotic downshock experiments were performed with cells that had accumulated radiolabelled substrate in the presence of 0.8 M KCl; these cells were diluted 5-fold with 50 mM potassium phosphate (pH 6.8)-5 mM $MgSO_4$ containing radiolabelled substrate (preheated at 30°C). Exchange reactions were assayed by allowing the cells to accumulate radiolabelled substrate, until steady state was reached (60-70 min), and, subsequently, competitors or inhibitors were introduced and samples were removed at time intervals and treated as described above. To monitor the effect of internal osmolyte pools on betaine and L-carnitine uptake, cells were loaded for given time periods with 1 or 2 mM unlabelled betaine or L-carnitine in the presence of 10 mM glucose in 50 mM potassium phosphate, pH 6.8, plus 5 mM $MgSO_4$. Cells were washed three times, and the transport of L-[^{14}C]carnitine and [^{14}C]betaine in these cells was determined as described above; the washing procedure did not result in large losses (less than 5%) of intracellular betaine or L-carnitine.

Chemicals. L-[N-*methyl*-^{14}C]carnitine (50-62 mCi/mmol) was from Amersham (Buckinghamshire, UK). Radiolabelled betaine was prepared enzymatically as described by Landfald and Strøm (16) from [N-*methyl*-^{14}C]choline (55 mCi/mmol, DuPont NEN) or [N-*methyl*-^{3}H]choline (60-90 Ci/mmol, DuPont, NEN). The identity of radiolabelled betaine was confirmed by thin-layer chromatography (24). Other chemicals were reagent grade and obtained from Sigma Chemical Company, St. Louis, Mo. or other commercial sources.

Results

Betaine and L-carnitine are handled by separate transport systems. Transport of [^{14}C]betaine and L-[^{14}C]carnitine was studied in the presence of 80-fold excess of unlabelled analogs (betaine, L-carnitine, L-proline, dimethylglycine or tetramethylglycine). Non-radioactive betaine, at 80-fold excess, completely inhibited [^{14}C]betaine uptake (1.3 mM, final concentration), whereas none of the other compounds had a significant effect on betaine

uptake. Similar results were obtained for the transport of L-[^{14}C]carnitine, which was only inhibited by a 80-fold excess unlabelled L-carnitine (data not shown).

Cells that were preloaded with [^{14}C]betaine lost the label upon addition of an 80-fold excess of unlabelled betaine (Fig. 1A). The potassium proton exchanger nigericin (2 μM) and the potassium ionophore valinomycin (1.5 μM), which in combination dissipate the proton (and, indirectly, the sodium) motive force, also caused exit of [^{14}C]betaine from preloaded cells. Addition of unlabelled L-carnitine to cells that had accumulated [^{14}C]betaine was without effect (Fig. 1A). Pre-accumulated L-[^{14}C]carnitine was not released upon addition of either unlabelled L-carnitine or unlabelled betaine, or the addition of nigericin plus valinomycin (Fig. 1B). These data are consistent with a secondary betaine transport system (8, 19) and a L-carnitine transport system that is dependent on ATP (28). Taken together, betaine and L-carnitine enter the cytoplasm solely via distinct transporters in *L. monocytogenes* Scott A.

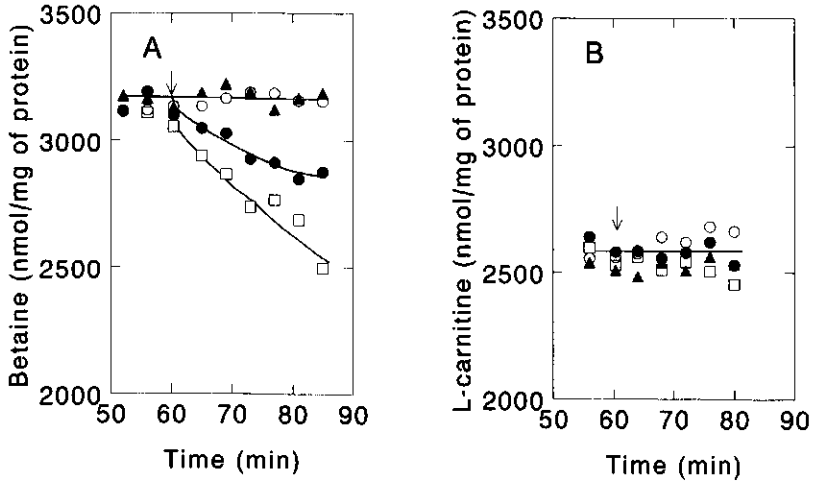

Figure 1. Betaine and L-carnitine efflux from *L. monocytogenes* Scott A. Cells were grown in DM with 0.3 M KCl, washed and resuspendend in 50 mM potassium phosphate (pH 6.9) containing 5 mM $MgSO_4$. Cells were energized with 10 mM glucose at 30°C and allowed to take up [^{14}C]betaine (panel A) or L-[^{14}C]carnitine (panel B) at high osmolarity (0.8 M KCl). Osmolytes were present at a final concentration of 1.3 mM. After 60 min of loading (indicated by *arrow*), a 80-fold excess of unlabeled betaine (●) or L-carnitine (▲), 1.5 μM valinomycin plus 2 μM nigericin (□), or an equivalent volume of water (○) was added.

Effect of osmolarity on betaine and L-carnitine uptake. Addition of increasing concentrations of KCl to the assay buffer (50 mM potassium phoshate - 5 mM $MgSO_4$) hardly influenced the initial rates of uptake of betaine and L-carnitine in *L. monocytogenes* Scott A grown in DM with 0.3 M KCl (Fig. 2). Lowering the osmolarity of the assay buffer by reducing the potassium phosphate concentration to 10 mM, 5 mM or 1 mM had no effect

either (data not shown). The final accumulation levels of both betaine and L-carnitine were dependent on the osmotic strenght; the effect was particularly pronounced in the case of betaine (Fig. 2). Comparable results were obtained when NaCl or sucrose were used to raise the osmolarity of the incubation mixture. The initial rates of betaine and L-carnitine uptake of cells grown in DM were similar as those of cells grown in DM with 0.3 M KCl, which also excludes significant induction of expression of the genes involved in the uptake of these compounds.

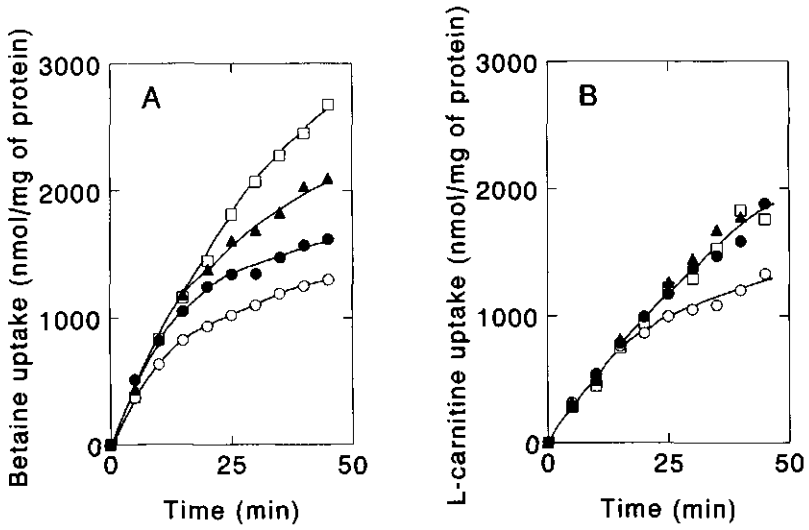

Figure 2. Effect of osmotic strength on betaine and L-carnitine uptake in *L. monocytogenes* Scott A. Cultures were grown in DM containing 0.3 M KCl. Transport assays of [^{14}C]betaine (panel A) and L-[^{14}C]carnitine (panel B) were performed at 30°C in 50 mM potassium phosphate (pH 6.9) containing 5 mM $MgSO_4$. After preenergization with 10 mM glucose for 5 min, KCl was added at varying concentrations together with the radiolabelled substrate (1.3 mM, final concentration). (○), no additions; (●), 0.15 M KCl; (▲), 0.3 M KCl; (□), 0.6 M KCl.

Kinetic properties of betaine uptake. Kinetic parameters of [^{14}]C-betaine uptake in *L. monocytogenes* Scott A grown in DM containing 0.3 M KCl were determined at substrate concentrations of 1-2000 μM in 50 mM potassium phosphate-5 mM $MgSO_4$. Michaelis-Menten and Eadie-Hofstee plots indicated an apparent K_m^{app} value of 10 $\pm$ 2 μM and a V_{max} of 56 $\pm$ 8 nmol min^{-1} mg protein^{-1}. Similar values were obtained under hyperosmotic conditions (50 mM potassium phosphate-5 mM $MgSO_4$ plus 0.6 M KCl). For comparison, L-carnitine is taken up with an apparent K_m^{app} of 10 $\pm$ 2 μM and a V_{max} of 48 $\pm$ 9 nmol min^{-1} mg protein^{-1} in *L. monocytogenes* Scott A (28), irrespective of the osmolarity of the medium.

Co-accumulation and transport of betaine and L-carnitine. To establish a possible pre-

ference in the accumulation of either betaine or L-carnitine, the intracellular concentrations were determined for cells grown in DM and DM plus 0.5 M NaCl containing betaine and/or L-carnitine. The addition of 1 mM betaine to DM resulted in an intracellular betaine concentration of 560 ± 60 nmol mg protein^{-1}, which increased to 1300 nmol ± 140 mg protein^{-1}, in the presence of 0.5 M NaCl (Table 1). The values obtained in the case of L-carnitine were 640 ± 70 nmol mg protein^{-1} and 990 ± 120 nmol mg protein^{-1}, respectively. When betaine and L-carnitine were supplied together, both at a concentration of 1 mM, betaine was preferentially accumulated, particularly in the high-osmolarity medium. No difference in the intracellular betaine and L-carnitine pools were detected when the available L-carnitine was reduced from 1 mM to 0.1 mM. However, a 10-fold reduction in the external betaine concentration (from 1 mM to 0.1 mM), with L-carnitine present at 1 mM, resulted in a substantial increase in the intracellular L-carnitine pool at the expense of intracellular betaine. Under these conditions the external betaine pool was not completely exhausted, since the maximal attainable intracellular betaine concentration at an external concentration of 0.1 mM is about 800 nmol mg protein^{-1}.

The regulation of betaine and L-carnitine accumulation in dependence of osmotic stress was further investigated by analyzing betaine and L-carnitine transport simultaneously in a dual label experiment. At low osmolarity, initial uptake rates of betaine and carnitine were similar, whereas in course of time betaine was accumulated to a higher level than L-carnitine (Fig. 3). Upon osmotic upshock, the initial rates of uptake remained the same (approximately 45 nmol min^{-1} mg of protein^{-1}), but the pools reached eventually increased. The increment in the final level of betaine was particularly prominent in the high osmolarity medium.

Table 1. Intracellular concentrations of osmolytes (nmol mg of protein^{-1}) in *L. monocytogenes* Scott A. Cells were grown until late-exponential growth phase in defined medium (DM), or in defined medium supplemented with 0.5 M NaCl (DMS), containing betaine and/or L-carnitine as indicated. Values are the mean of triplicate samples of independent cultures.

	DM-grown		DMS-grown	
Medium additions	[Betaine]	[L-carnitine]	[Betaine]	[L-carnitine]
No additions	0	0	0	0
1 mM B[a]	560 ± 60	0	1300 ± 140	0
1 mM C[b]	0	640 ± 70	0	990 ± 120
1 mM B + 1 mM C	490 ± 50	240 ± 30	1230 ± 140	110 ± 20
1 mM B + 0.1 mM C	490 ± 50	220 ± 30	1350 ± 140	60 ± 10
0.1 mM B + 1 mM C	200 ± 30	400 ± 50	540 ± 60	460 ± 50

[a] B, betaine
[b] C, L-carnitine

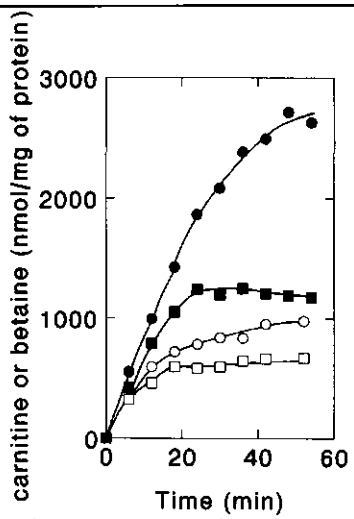

Figure 3. Simultaneous uptake of [^{3}H]betaine and L-[^{14}C]carnitine under low and high osmolarity conditions. Cells were grown in DM containing 0.3 M KCl, washed and resuspendend in 50 mM potassium phosphate (pH 6.9) containing 5 mM $MgSO_4$. After 5 min of preincubation with 10 mM glucose at 30°C, [^{3}H]betaine plus L-[^{14}C]carnitine (final concentrations of 1.3 mM) were introduced with (●,■) or without 0.8 M KCl (○,□). Transport of [^{3}H]betaine (○,●) and L-[^{14}C]carnitine (□,■) is depicted.

Internal betaine and L-carnitine pools at different external osmolarities. In an earlier study (28), we observed that growth in the presence of L-carnitine or betaine reduced the rate of L-carnitine transport, which could be explained by an inhibition of the L-carnitine transport system by intracellular betaine and L-carnitine. These considerations, together with the apparent lack of a direct effect of osmolarity on carrier activity, prompted us to study the regulation of the L-carnitine and betaine tranport systems by internal (preaccumulated) substrate in detail. To put this into effect, cells were grown in DM with 0.3 M KCl lacking betaine and L-carnitine, collected at late-exponential growth phase, washed two times and subsequently incubated with 1 mM betaine or 1 mM L-carnitine for different periods of time in the presence of glucose. Following the removal of external betaine or L-carnitine, the initial rate of [^{14}C]betaine uptake as a function of internal osmolyte concentration was determined. As shown in Fig. 4, the rate of uptake of betaine (A) and L-carnitine (B) decreased with increasing internal amounts of betaine and L-carnitine. This suggests that both transporters are subject to inhibition by preaccumulated betaine and L-carnitine, and that betaine as well as L-carnitine are equally effective in inhibiting the activity of the transporters at the inner site of the cytoplasmic membrane.

Next, cells that had been preloaded with unlabelled betaine or L-carnitine for 30 min, resulting in internal concentrations of approximately 350 mM, were used to study [^{14}C]betaine and L-[^{14}C]carnitine uptake at varying external osmolarities. Without osmotic upshock, the uptake rates of [^{14}C]betaine and L-[^{14}C]carnitine in cells preloaded with betaine or L-carnitine, respectively, were low (about 10 nmol min^{-1} mg $protein^{-1}$) (Fig. 5). A significant

increase in the initial rates of uptake of both [^{14}C]betaine and L-[^{14}C]carnitine transport was encountered upon imposition of hyperosmotic conditions of cells pre-incubated with betaine and L-carnitine, respectively (Fig. 5). Similar results were obtained when NaCl instead of KCl was used to raise the osmolarity of the assay mixture. The insets of the figures show that hyperosmotic conditions have no effect on the initial rate of uptake when betaine or L-carnitine are not present intracellularly. These data suggest an involvement of membrane tension or turgor pressure in the activity of the transporters, which seems to exert its effect on the interaction of the ligand (substrate) with the carrier protein at the *trans* site of the membrane. As anticipated, the final accumulation levels of radiolabelled betaine and L-carnitine were higher in control cells than in cells that had preaccumulated non-labelled betaine or L-carnitine, and both subjected to an osmotic upshock (not shown).

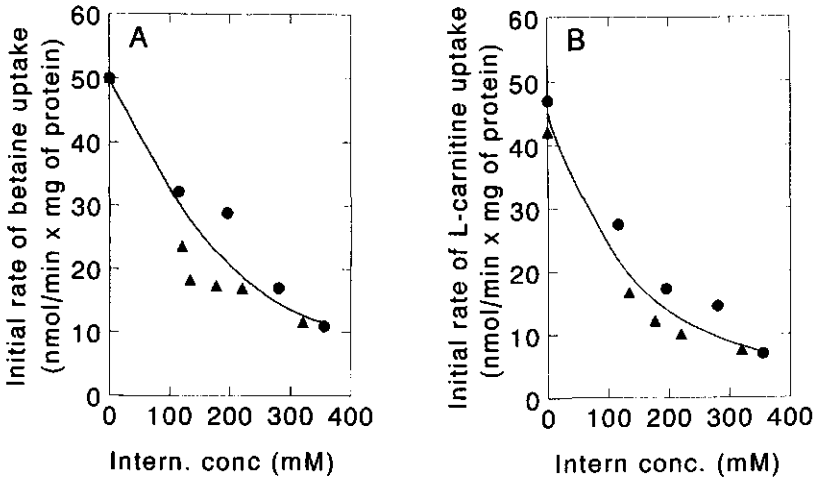

Figure 4. Rates of betaine and L-carnitine uptake at different internal osmolyte concentrations. Cells grown in DM with 0.3 M KCl were washed and resuspended in 50 mM potassium phosphate (pH 6.9) containing 5 mM $MgSO_4$. To establish different internal osmolyte concentrations, the cells were preloaded at 30 °C in the presence of 1 mM betaine or L-carnitine and 10 mM glucose as energy source. The rate of [^{14}C]betaine and L-[^{14}C]carnitine uptake was estimated from the uptake for 10 min in the presence of 10 mM glucose and after removal of unlabelled substrate. Symbols: (●), internal betaine concentration; (▲), internal L-carnitine concentration.

Betaine and L-carnitine exit upon osmotic downshock. When glucose metabolizing cells, that had accumulated betaine or L-carnitine, were subjected to an osmotic downshock, a rapid efflux of both osmolytes was observed (Fig. 6). These fast effluxes of betaine and L-carnitine were completed within 5 sec. In addition, a second slower phase of efflux was noticable, which was particularly prominent for betaine.

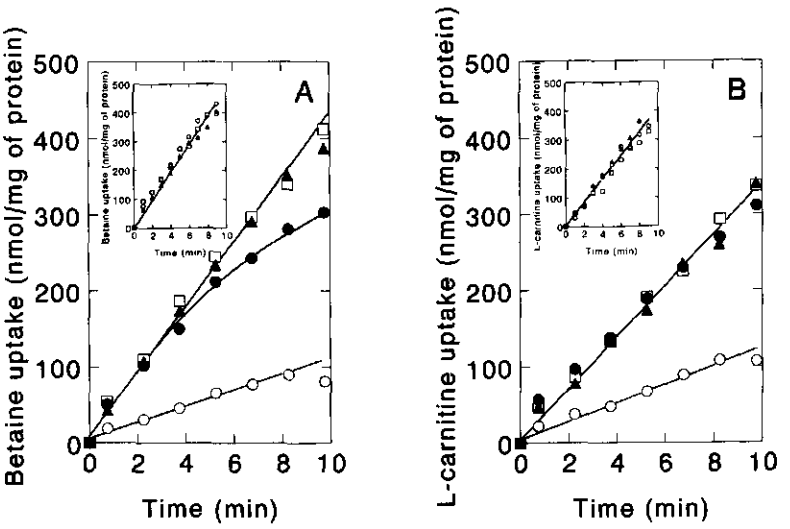

Figure 5. Effect internal osmolytes and hyperosmotic conditions on transport of [^{14}C]betaine and L-[^{14}C]carnitine in *L. monocytogenes* Scott A. Cells were grown in DM with 0.3 M KCl, washed and resuspendend in 50 mM potassium phosphate (pH 6.9) containing 5 mM $MgSO_4$. Aliquots of cells were preloaded with unlabelled betaine or L-carnitine for 30 min as described in the legend of Fig. 4. Following the removal of unlabelled substrate, uptake of radiolabelled substrates was assayed at varying external osmolarities. Uptake of [^{14}C]betaine was conducted with cells preincubated with betaine (panel A) and uptake of L-[^{14}C]carnitine was performed with cells preincubated with L-carnitine (panel B). In the insets the uptake rates of betaine and L-carnitine in non-loaded cells are shown for comparison. Symbols: (○), no additions; (●), 0.15 M KCl; (▲), 0.3 M KCl; (□), 0.6 M KCl.

Discussion

In this study, it was shown that *L. monocytogenes* Scott A possesses highly specific and separate transport systems for the osmolytes betaine and L-carnitine. The two transporters have a comparable high affinity for their substrates (apparent K_ms of about 10 μM). These findings are unique as both transporters have immense scavenging capacity for their substrate, and specific independent transport systems for betaine and carnitine have not been described for other microorganisms. In *L. plantarum*, betaine and L-carnitine enter the cytoplasm via the same transport system with high affinity for both substrates (9), and in *E. coli*, carnitine is transported via ProP and ProU, which are driven by a sodium motive force and ATP, respectively (5, 27).

In a previous study, we demonstrated that in *L. monocytogenes* Scott A, L-carnitine is taken up via an ATP-dependent transporter (28). In a recent study by Gerhardt *et al.* (8), evidence was obtained for the involvement of a sodium motive force as driving force for betaine uptake in *L. monocytogenes* DP-L1044. The addition of unlabelled betaine to *L. monocytogenes* Scott A, preloaded with [^{14}C]betaine, resulted in exit of radioactive label which is thought to be due to exchange of internal for external betaine. Moreover, dissipation

of the electrochemical ion gradients by nigericin plus valinomycin to cells that had preaccumulated [^{14}C]betaine caused efflux of label driven by the betaine concentration gradient. These results support a secondary transport mechanism for betaine uptake in *L. monocytogenes* Scott A as these systems generally operate in two directions in contrast to ATP-dependent solute uptake systems.

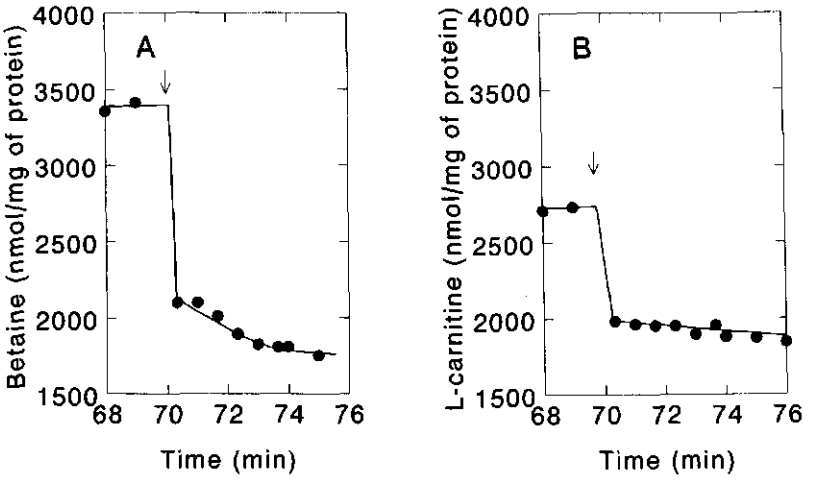

Figure 6. Betaine and L-carnitine efflux from *L. monocytogenes* Scott A in response to osmotic downshock. Cells grown in DM with 0.3 M KCl were washed and resuspended in 50 mM potassium phosphate (pH 6.9)-5 mM $MgSO_4$. After 5 min of preenergization with 10 mM glucose at 30°C, uptake was initiated by addition of 1.3 mM [^{14}C]betaine (panel A) or 1.3 mM L-[^{14}C]carnitine (panel B) together with KCl (final concentration of 0.8 M). After 70 min of uptake, samples were diluted 5-fold with 50 mM potassium phosphate (pH 6.9) containing 5 mM $MgSO_4$ plus 1.3 mM [^{14}C]betaine (A) or 1.3 mM L-[^{14}C]carnitine (B).

L. monocytogenes cultured in DM supplemented with 1 mM betaine plus 1 mM L-carnitine, accumulated a higher intracellular level of betaine than L-carnitine (about two-fold). The preferred accumulation of betaine was even more prominent in a high-osmolarity environment. From the dual label uptake experiment, it could be inferred that the initial uptake rates of betaine and L-carnitine at "zero" *trans* substrate concentrations are not affected by the presence of the other substrate (see Fig. 7). Moreover, at "zero" *trans* substrate concentrations, the rate of uptake was not affected by an osmotic upshock (insets Fig. 5). The final levels of accumulation of betaine and L-carnitine were influenced by the medium osmolarity, and it was demonstrated that regulation of both betaine and L-carnitine uptake is subject to inhibition by preaccumulated solute. Internal betaine not only inhibited the transport of external betaine but, additionally, it inhibited the transport of external L-carnitine. Accordingly, internal L-carnitine inhibited both betaine and L-carnitine transport. This represents a novel mechanism of regulation of compatible solute accumulation, *i.e.*, both

transporters are inhibited by the presence of compounds on the inner surface of the membrane which are not recognized by the respective transporter at the outer surface of the membrane. The inhibition of betaine and L-carnitine transport by preaccumulated solute was relieved when cells that had been preloaded with betaine or L-carnitine where subjected to an osmotic upshock. This is most likely a consequence of an altered membrane structure due to changes in membrane tension affecting conformation of the transporters. In kinetic terms it could mean that upon osmotic upshock the affinity of the transporters for betaine and L-carnitine at the inner surface of the membrane is lowered (Fig. 7). The observation that the L-carnitine transport system is inhibited stronger by internal betaine or L-carnitine (50% inhibition at 110 mM) than the betaine transport system (50% inhibition at 175 mM), may explain the preferred uptake of betaine over L-carnitine.

The initial rates of uptake in preloaded cells were similar irrespective of whether the osmotic strength was raised by 0.15, 0.3 or 0.6 M KCl (or NaCl) and were comparable to those obtained with non-loaded cells under the same osmotic stress conditions. These data may explain why Patchett *et al.* (19) observed much lower betaine uptake rates in *L. monocytogenes* NCTC 7973 (5 to 10 times lower) than we did in *L. monocytogenes* Scott A. Their studies were conducted with cells grown in broth, which is known to contain significant amounts of betaine and carnitine (12, 13, 20, 23, 25). Accumulation of these compatible solutes during growth could have resulted in the reduced uptake rates for betaine. The approximate 10-fold increased uptake rate in the presence of NaCl is consistent with the regulation of activity by an osmotic upshock as described in this paper.

When *L. monocytogenes* is faced with a decrease in the external osmolarity, *i.e.* a hypotonic osmotic shock, an immediate release (within 5 seconds) of the osmolytes betaine and L-carnitine was observed. The rates of efflux were much higher than the rates of uptake, which suggests that this rapid efflux of betaine and L-carnitine is mediated via a channel-like activity rather than via a carrier protein (1, 9). The rapid osmolyte release upon osmotic downshock was followed by a slower second phase of efflux, which was particularly significant in the case of betaine. This is most likely due to downhill efflux of [^{14}C]betaine via the same carrier protein that is responsible for the uptake of betaine. A similar pattern of biphasic efflux of betaine was reported for *L. plantarum* cells that were loaded with [^{14}C]betaine. In this organism, betaine is taken up via a kinetically irreversible ATP-dependent transport system and a distinct uniport system was proposed for the slow phase of betaine efflux upon an osmotic downshock (9).

The experiments presented in this paper also show that *L. monocytogenes* not only establishes substantial internal concentrations of betaine and L-carnitine when grown at high osmolarity (*i.e.* DM containing $\geq$ 0.15 M NaCl or KCl), but also accumulates significant amounts of betaine and L-carnitine when grown in DM (0.4 osmol/kg) without added NaCl or KCl. Accumulation of compatible solutes in the absence of osmotic stress, has also been described for other Gram-positive bacteria. This is thought to be a direct consequence of their maintenance of a relatively high turgor pressure in comparison to Gram-negative bacteria (4, 10, 25, 29).

The most important findings of the present study relate to the osmotic regulation of

betaine and L-carnitine uptake which are summarized in Fig. 7. Different conformations of the putative betaine and L-carnitine uptake systems are depicted for low and high osmolarity media and under conditions that the intracellular concentration of betaine and/or L-carnitine are low and high; the thickness of the arrows reflects the relative activity of the transport systems under the varying conditions. Upon a hyperosmotic shock, betaine and L-carnitine transport in *L. monocytogenes* Scott A is stimulated in proportion to the *trans* substrate concentration. At zero *trans* substrate concentration the activity is already maximal and not affected by an osmotic upshock. The osmotic regulation of betaine and L-carnitine transport in *L. monocytogenes* is different from that in *L. plantarum*, where an osmotic upshock activates the system irrespective of the internal substrate concentration (9).

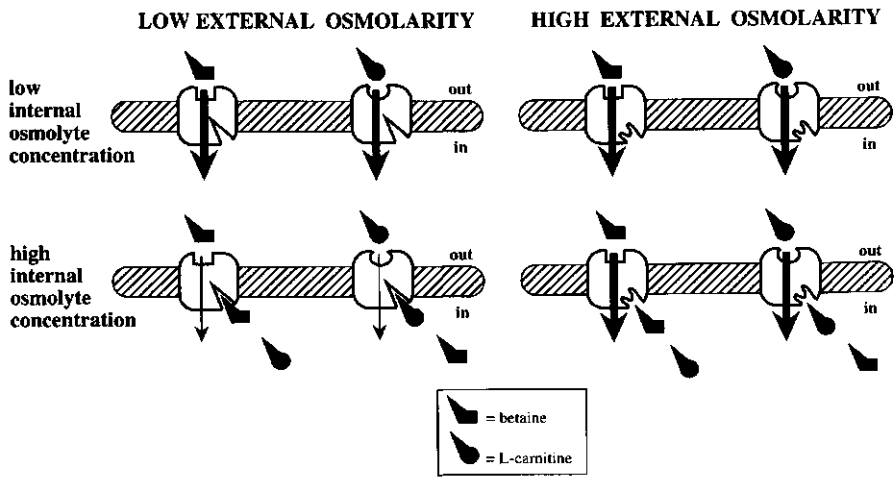

Figure 7. Model for osmotic regulation of betaine and L-carnitine uptake in *L. monocytogenes* Scott A. The relative uptake activities are indicated by the thickness of the arrows. For both transport systems, betaine can replace L-carnitine at the *trans* side of the cytoplasmic membrane.

Acknowledgements

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7

A possible role of ProP, ProU, and CaiT in osmoprotection of *Escherichia coli* by carnitine

Annette Verheul, Jeroen A. Wouters, Frank M. Rombouts and Tjakko Abee

Abstract

Exogenously provided carnitine (ß-hydroxy-L-τ-*N*-trimethyl aminobutyrate) was found to stimulate aerobic growth of enterohaemorrhagic *Escherichia coli* O157:H7 in a medium of inhibitory osmotic strength. Its osmoprotective ability is comparable with that of betaine. Since carnitine is an important compound in mammalian tissues it is suggested that it might play a role in the growth of the pathogen on low a_w meat products. Using specific uptake mutants of *E. coli* K-12, it was established that under osmotic stress carnitine accumulates in the cytoplasm following import through the ProP and ProU transport systems. Betaine and carnitine also protect *E. coli* cells while growing anaerobically at inhibitory osmolarity. Under these conditions, an *E. coli* K-12 strain with lesions in both *proP* and *proU*, accumulates low levels of L-carnitine but fails to accumulate betaine when these compounds are supplied in the external medium. This is probably a result of uptake of L-carnitine by the secondary transporter CaiT. The *caiT* gene forms part of the *caiTABCDE* operon which encodes the carnitine pathway, and is transcribed during anaerobic growth in the presence of carnitine. However, further experiments revealed that the carnitine pathway, including CaiT, does not play a significant role in osmoregulation of *E. coli* during anaerobiosis. Together, the results indicate that ProP and ProU are the sole transport systems involved in carnitine influx both in aerobically and anaerobically osmotically stressed *E. coli* cells.

This chapter has been submitted for publication to Appl. Environ. Microbiol.

Introduction

Since initial identification of *Escherichia coli* O157:H7 as the cause of outbreaks of hemorrhagic colitis in 1982 (30), many subsequent outbreaks, often associated with eating undercooked beef have been reported (1, 10). *E. coli* O157:H7 does not survive at an a_w of < 0.95 (ca. 8.5% NaCl) (32), however, a 1994 outbreak of hemorrhagic colitis caused by *E. coli* O157:H7 implicated dry-cured salami (2), a product with an a_w < 0.9 (26), as the vehicle of illness. As previously observed with various strains of *E. coli*, growth of *E. coli* O157:H7 at high osmolarity might involve the accumulation of external osmolytes such as betaine and proline (7, 25, 33).

Many studies have been devoted to osmoregulation in *E. coli*. The first adaptative response to elevated medium osmolarity is the accumulation of high intracellular levels of K^+ and glutamate (12). In a later phase of adaptation, the osmoprotectant trehalose is synthesized (14), which substitutes for potassium glutamate (9). In the presence of proline or betaine, *E. coli* preferentially accumulates these compounds and trehalose accumulation is diminished (25, 33). Uptake of proline in *E. coli* is mediated by three transporters: PutP, ProP and ProU. The PutP system is required for the transport of proline when this compound is used as a carbon or nitrogen source; it is not important for the transport of proline as an osmoprotectant (18). The ProP and ProU systems have been shown to be responsible for the accumulation of proline and betaine under conditions of hyperosmolarity. The ProP system functions as a constitutive sodium-symporter which undergoes 2- to 3-fold stimulation by growth at high osmolarity (6, 7). The ProU system belongs to the ATP-driven binding-protein dependent transport systems. ProU-mediated betaine transport is highly osmotically stimulated at the level of gene expression. The ProP system has a K_m of 0.3 mM for proline and a K_m of 44 μM for betaine, for ProU these values are 0.2 mM and 1.4 μM, respectively (7).

Recently, it was demonstrated that L-carnitine (ß-hydroxy-L-τ-*N*-trimethyl aminobutyrate) is involved in osmoprotection of the gram-positive bacteria *Lactobacillus plantarum* (23) and *Listeria monocytogenes* (3, 35). L-carnitine is a ubiquitously occurring substance in biological material due to its crucial function in the ß-oxidation of fatty acids in mitochondria (4). Consequently, L-carnitine is always abundant in meat tissues which invokes questions on the role of carnitine in the osmotic stress response of *E. coli* O157:H7. Like other members of the *Enterobacteriaceae* family, *E. coli* is able, during anaerobic growth and in the presence of carbon and nitrogen sources, to catalyze the dehydration of L-carnitine into crotonobetaine and the subsequent reduction into τ-butyrobetaine as the final product. The biological significance of the carnitine pathway is thought to lie in the transfer of redox equivalents to crotonobetaine in the absence of other electron acceptors. Recently, the complex *caiTABCDE* operon encoding six proteins for the carnitine pathway in *E. coli* was characterized, CaiT is the proton motive force driven transport system for carnitine in *E. coli* (11, 22). Transcription of the *cai* operon is induced during anaerobic growth in the presence of L-carnitine, the induction of L-carnitine transport is partially or completely repressed in the presence of either τ-butyrobetaine, glucose, nitrate or O_2. The CaiT system has a K_m of 0.53

mM for L-carnitine and shows considerable affinity for other trimethylamine carboxylic molecules like D-carnitine, crotonobetaine and betaine (11, 22).

The aim of the present study was to obtain information on the role of carnitine in the osmotic stress response of *E. coli* and to assess the involvement of ProP, ProU, CaiT and possible other transporters herein (using wild-type and transport mutants) both under aerobic and anaerobic conditions.

Materials and Methods

Bacterial strains. Bacterial strains used in this study were *E. coli* O157:H7 ATCC 43895, *E. coli* K-12 (wild-type, WT) and two derivatives of *E. coli* K-12: WG244 (F⁻ *trp lacZ rpsL thi Δ(putPA)101 proP219 zjd351*::Tn*10*) and CLG85 (F⁻ *trp lacZ rpsL thi Δ(putPA) 101 proP219 proU*::Tn*phoA zfh1*::Tn*10*), which were kindly provided by Dr. Gutierrez (Laboratoire de Microbiologie et Génétique Moléculaire du CNRS, Toulouse, France). The DNA of *E. coli* O157:H7 was screened for the presence of the *proP* gene (8) by PCR with primers (5'-TTTCCCGGGGGCTGACCC-3') and (5'GTTGCTGCACAGGCGGG-3'). The presence of *proU* (17) in *E. coli* O157:H7 was checked with (5'-TTGAACCCACCCGCGGGC-3') and (5'-GCACAGGGTGCCTGTCCG-3'). The predicted sizes of the fragments are 1.4 kb and 0.9 kb, respectively. The wild-type *E. coli* K-12 strain was taken along as a control. PCRs were carried out in standard PCR buffer with 250 μM deoxynucleoside triphosphates, 0.2 μM primers, and 1 U of *Taq* polymerase (Gibco) in a total volume of 50 μl (30 cycles of 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C).

Growth protocols. Cells were grown at 37°C in a minimal medium (MM) containing 46 mM $Na_2HPO_4.2H_2O$, 23 mM $NaH_2PO_4.H_2O$, 8 mM $(NH_4)_2SO_4$, 1 mM trisodium citrate, 5 mM KCl, 0.4 mM $MgSO_4.7H_2O$, 6 μM $FeSO_4.7H_2O$, 10 mg/l thiamine, 50 mg/l L-tryptophan, and 0.2 ml/l of a trace elements solution (37). The minimal medium was supplemented with glucose (10 mM) or glycerol (50 mM), sodium fumarate (18 mM) and casamino acids (0.1% (wt/vol)) (Difco, Detroit, Mich.) as indicated. NaCl was added at different concentrations to increase medium osmolarity, betaine, DL-carnitine and L-proline were supplied at a concentration of 1 mM.

Growth under aerobic conditions was in 50 or 100 ml cultures in 300-ml Erlenmeyer flasks shaken at 200 rpm. Anaerobic cultivation took place in tightly stoppered 100-ml bottles, in which the remaining headspace was evacuated several times and refilled with nitrogen until equilibrium was achieved. For anaerobic growth curve measurements, 10-ml calibrated glass tubes were completely filled with medium in an anaerobic hood. Cysteïne.HCl (0.5 g/l) was added to remove any remaining oxygen and resazurin indicator (1 mg/l) was used to check anaerobiosis. Growth curves were obtained by subculturing overnight cultures into fresh medium using an inoculum size of 1% (vol/vol) and monitoring optical density (Pharmacia LKB Novaspec II) at 620 nm (OD_{620}). The values reported for the 10-ml calibrated glass tubes have been normalized for a 1 cm light path. Uptake assays and determination of intracellular solute content were conducted on cells in the late-exponential growth phase after growth under the appropriate conditions.

Transport protocols. Cells were harvested by centrifugation (11000 x g, 10 min, 10°C), washed twice and resuspended in 50 mM potassium phosphate (pH 6.9) with 5 mM $MgSO_4$ containing 50 μg of chloramphenicol per ml and stored on ice until use. The NaCl concentration of the buffer equaled the NaCl concentration of the culture medium. Measurements of L-[^{14}C]carnitine and L-[^{14}C]proline uptake were carried out at 37°C in glucose-energized cells as described by Verheul *et al.* (35). For cells grown anaerobically, the buffer was degassed with nitrogen and cysteine.HCl (5 g/l) was

included in the buffer. The L-[^{14}C]carnitine uptake assays were performed under a constant stream of nitrogen gas in the presence of 50 mM glycerol and 18 mM sodium fumarate. The specific activity of the radiolabeled compounds was varied by mixing with unlabelled compound. The protein content was determined by the Lowry method using bovine serum albumin as a standard (27).

Extraction and identification of cytoplasmic solutes. Cells were harvested by centrifugation (11000 x g, 10 min, 10°C) and washed twice in 50 mM potassium phosphate (pH 6.9)-5 mM $MgSO_4$ isotonic with the growth medium. Cells were freeze-dried, and subsequently compatible solutes were extracted using a technique essentially described by Bligh and Dyer (5) modified by Galinski and Herzog (13). High performance liquid chromatography (HPLC) detection of L-proline and trimethylammonium compounds was performed on a LiChrospher 100 NH_2, 5 μm column (Merck, Darmstadt, Germany) at a flow rate of 1 ml/min at 45°C with a mobile phase of 80:20 (vol/vol) acetonitril-20 mM potassium phosphate (pH 7.0). Separated components were identified by a refractive index detector (Separations, Spark, Holland) set at a wavelength of 190 nm. For trehalose determination, high performance anion-exchange chromatography was employed on a 4 x 250 mm Dionex (Sunnyvale, CA) CarboPac PA1 column connected with a Dionex pulsed electrochemical detector. Elution was at room temperature with a NaOH/water gradient (1 ml/min) starting with 5% 0.1 M NaOH for 3 min followed by a gradient to 55% 0.1 M NaOH for another 17 min. Standard solutions of proline, betaine, carnitine and trehalose were used for calibration. Amino acids were analyzed using HPLC as described previously (34).

Chemicals. L-[*N-methyl*-^{14}C]carnitine (1.96 TBq/mol) was purchased from Dupont, [^{14}C]proline (9.62 TBq/mol) was from Amersham. All other chemicals were of commercially available analytical grade.

Results

Growth stimulation of *E. coli* O157:H7 by carnitine at high osmolarity. The growth of *E. coli* O157:H7 in MM with glucose was severely reduced when the osmolarity of the medium was raised by adding NaCl to a final concentration of 0.5 M (Fig. 1). The addition of either 1 mM betaine or 1 mM DL-carnitine to this high osmolarity growth medium stimulated the growth of *E. coli* O157:H7 equally well, which suggests that carnitine is as efficient as betaine for osmoprotection. To gain insight in the route of carnitine uptake in *E. coli* O157:H7, we aimed to use derivates of this strain with ProP and ProU deletions. Since these were not readily available, a set of *E. coli* K-12 strains was used for this purpose, which are listed in Table 1. The presence of *proP* and *proU* in *E. coli* O157:H7 was verified with specific PCR amplifications (data not shown).

Effect of osmoprotectants on growth of *E. coli* K-12 strains at high osmolarity. (*i*) aerobic growth. Growth of the WT and transporter mutants was significantly affected by the addition of 0.5 M NaCl to the medium (Fig. 2). The growth of WT and WG244 was improved by the presence of betaine and proline in medium of inhibitory osmolarity. Carnitine was almost as effective as betaine in promoting growth of WT and WG244 at 0.5 M NaCl, which agrees with the results obtained for *E. coli* O157:H7 (Fig. 1). The osmotic inhibition of growth in the mutant lacking PutP, ProP and ProU (*i.e.* CLG85) could not be relieved by betaine, proline or carnitine (Fig. 2C).

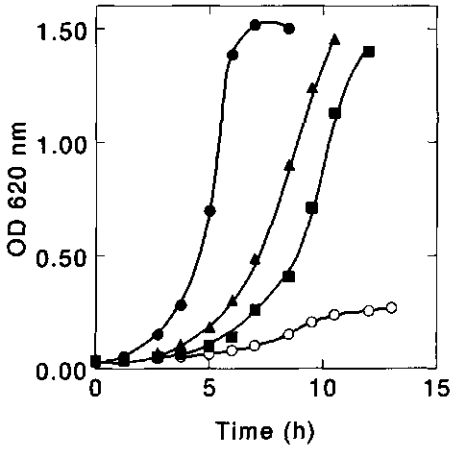

Figure 1. Growth stimulation of *E. coli* O157:H7 at high osmolarity by betaine and DL-carnitine. *E. coli* O157:H7 was grown aerobically in MM containing 10 mM glucose with no NaCl (●), 0.5 M NaCl (○), 0.5 M NaCl and 1 mM betaine (▲), or 0.5 M NaCl and 1 mM carnitine (■).

Table 1. Genotypes of *E. coli* K-12 strains

Strain	Relevant genotype	Transport systems			
		PutP[a]	ProP[b]	ProU[c]	CaiT[d]
WT K-12	Wild Type	+	+	+	+
WG244	F⁻ *trp lacZ rpsL thi* Δ*(putPA)101 proP219 zjd351*::Tn*10*	-	-	+	+
CLG85	F⁻ *trp lacZ rpsL thi* Δ*(putPA)101 proP219 proU*::Tn*phoA zfh1*::Tn*10*	-	-	-	+

[a] PutP is constitutive and transports proline for use as carbon or nitrogen source
[b] ProP is constitutive and 2- to 3-fold induced during growth at high osmolarity and activated by osmotic stress
[c] ProU is induced during growth at high osmolarity and activated by osmotic stress
[d] CaiT is induced during anaerobic growth in the presence of carnitine

***(ii)* anaerobic growth.** Increasing the osmolarity of the growth medium by adding 0.5 M NaCl during anaerobic growth of WT and WG244 in MM with 10 mM glucose severely inhibited growth (Fig. 3A,B). The osmotic inhibition of growth could not be diminshed by the addition of 1 mM proline. However, both betaine and carnitine were found to be capable

of promoting the growth of osmotically stressed *E. coli* WT and WG244 under anaerobic conditions; betaine was a more potent osmoprotectant than carnitine under these conditions for both strains. CLG85 showed hardly any growth in MM containing 10 mM glucose under anaerobic conditions and growth was completely inhibited upon addition of 0.5 M NaCl to this medium, regardless of the presence of L-proline, betaine or DL-carnitine (Fig. 3C). The results demonstrate that carnitine can serve as an osmoprotectant both in aerobically and anaerobically grown *E. coli* K-12 strains containing ProU (WG244 strain) or ProP and ProU (WT strain).

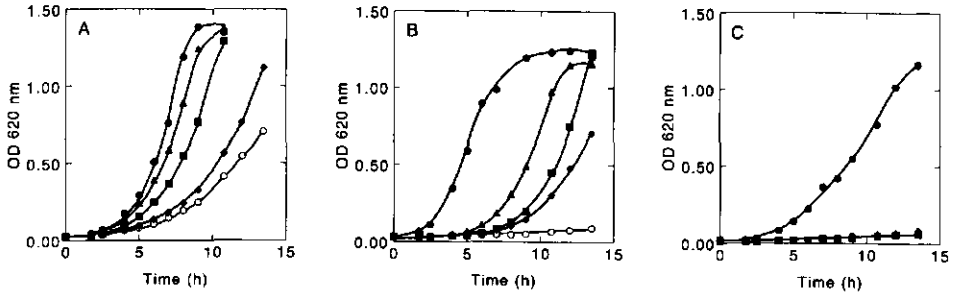

Figure 2. Effect of proline, betaine and DL-carnitine on aerobic growth of *E. coli* strains WT (panel A), WG244 (panel B) and CLG85 (panel C) in high osmolarity MM containing 10 mM glucose. The osmolarity of the medium was increased by adding 0.5 M NaCl. The optical density at 620nm was monitored in medium containing (●), no NaCl; (○) NaCl; (◆), NaCl and 1 mM proline, (▲), NaCl and 1 mM betaine or (■) NaCl and 1 mM DL-carnitine.

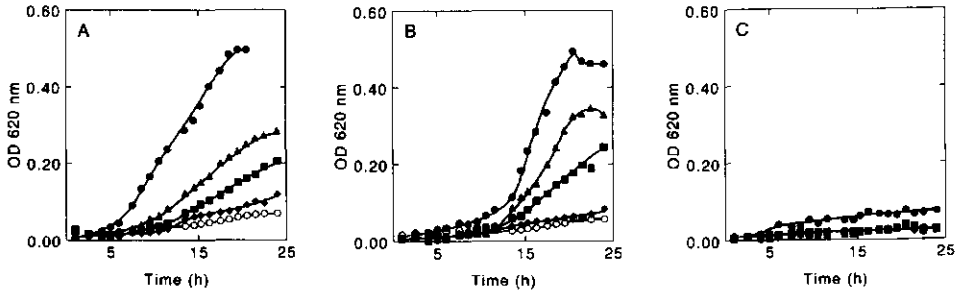

Figure 3. Effect of proline, betaine and DL-carnitine on anaerobic growth of *E. coli* strains WT (panel A), WG244 (panel B) and CLG85 (panel C) in high osmolarity MM containing 10 mM glucose. The osmolarity of the medium was increased by adding 0.5 M NaCl. The optical density at 620nm was monitored in medium containing (●), no NaCl; (○) NaCl; (◆), NaCl and 1 mM proline, (▲), NaCl and 1 mM betaine or (■) NaCl and 1 mM DL-carnitine.

Osmoprotectant accumulation in *E. coli* K-12 strains during aerobic growth. Wild-type *E. coli* and its derivatives WG244 and CLG85 were evaluated for their abilities to accumulate

osmolytes in the absence or presence of osmotic stress (0.5 M NaCl) under aerobic conditions. Without NaCl, no accumulation of betaine, proline, carnitine or trehalose was detected in any of the strains and the amino acid levels were 20-25 μmol g dry weight^{-1} for all three strains under these conditions (data not shown). In the high osmolarity medium without added osmoprotectants, no accumulation of proline, betaine or carnitine was seen in any of the strains (Table 2) which excludes *de novo* synthesis of these substances. However, WT synthesized a substantial amount of trehalose (218 $\pm$ 49 μmol g dry weight^{-1}) in response to high osmolarity, whereas WG244 synthesized only 20 $\pm$ 5 μmol trehalose per g dry weight under these conditions (Table 2). The intracellular amino acid pools in osmotically stressed WT and WG244 were 75 $\pm$ 10 and 70 $\pm$ 10 μmol g dry weight^{-1}, respectively (Table 2), which represents approximately a 3-fold increase in comparison with the amino acid content in non-stressed cells. As presented in Fig. 2C, CLG85 showed no growth in the high osmolarity growth medium, however, after approximately 25 h, CLG started to grow in all NaCl containing media with comparable growth rates (approximately 0.05 h^{-1}), the final OD_{620} reached in the four media was about 0.8 (not shown). No accumulation of L-proline, betaine or carnitine was detected in these cells (Table 2), a significant amount of trehalose was found in CLG85 grown in high osmolarity MM regardless of the presence of osmoprotectants.

Supplementation of 1 mM betaine to the high osmolarity medium resulted in an intracellular betaine concentration of 608 $\pm$ 42 μmol g dry weight^{-1} for WT, WG244 accumulated 487 $\pm$ 47 μmol g dry weight^{-1} under equal conditions. In the case of exogenously added carnitine, intracellular values were 470 $\pm$ 30 μmol g dry weight^{-1} for WT and 407 $\pm$ 64 μmol g dry weight^{-1} for WG244. For L-proline the values amounted to 210 $\pm$ 35 μmol g dry weight^{-1} and 188 $\pm$ 33 μmol g dry weight^{-1} for WT and WG244, respectively (Table 2). Trehalose synthesis in both strains was suppressed in the presence of exogenous osmolytes and the amino acid content was reduced to the levels found in non-osmostressed cells, *i.e.* 20-25 μmol g dry weight^{-1} (Table 2). The results show that carnitine is a substrate for ProU but give no decisive answer for ProP since in its absence, the carnitine level was not decreased to a great extent (WT versus WG244). The finding that the mutant defective in ProP and ProU (CLG85) accumulates none of these osmolytes at high osmolarity excludes the involvement of transporters other than ProP and ProU in osmoregulation of *E.coli* K-12 under aerobic conditions.

Osmoprotectant accumulation in *E. coli* K-12 strains during anaerobic growth. Since CLG85 showed no growth even in the low osmolarity situation when grown anaerobically, another medium was selected in order to monitor the accumulation levels under anaerobic growth conditions in the *E. coli* K-12 strains. Moreover, CaiT induction in anaerobically grown *E. coli* is repressed in the presence of glucose (22) and therefore glucose was replaced by glycerol, since our interest is in the possible involvement of CaiT in osmotic stress-induced carnitine accumulation under anaerobic conditions. Additionly, fumarate was added as electron acceptor to prevent dehydrogenation of L-carnitine into crotonobetaine and subsequent reduction to γ-butyrobetaine (22). Casamino acids (0.1% (wt/vol)) were included

Table 2. Accumulation of osmoprotectants (μmol/g dry weight) in *E. coli* K-12 strains grown aerobically at high osmolarity[a]

strain, medium additions	betaine	carnitine	proline	amino acids	trehalose
WT					
none	0[b]	0	0	75 ± 10	218 ± 49
betaine	608 ± 42	0	0	20 ± 5	0
carnitine	0	470 ± 30	0	25 ± 5	0
proline	0	0	210 ± 35	nd[c]	0
WG244					
none	0	0	0	70 ± 10	20 ± 5
betaine	487 ± 47	0	0	20 ± 5	0
carnitine	0	407 ± 64	0	25 ± 5	0
proline	0	0	188 ± 31	nd	0
CLG85					
none	0	0	0	40 ± 6	165 ± 25
betaine	0	0	0	25 ± 5	172 ± 31
carnitine	0	0	0	25 ± 5	160 ± 16
proline	0	0	0	nd	nd

[a] Cultures were grown at 37°C in MM plus 10 mM glucose plus 0.5 M NaCl and 1 mM betaine, 1 mM D,L-carnitine or 1 mM L-proline where indicated until late-exponential growth phase. Cells were washed, cytoplasmic solutes were extracted and quantified by HPLC. Values are the mean of triplicate samples of independent cultures.

[b] 0 indicates below detection limit;

[c] nd, not determined

Table 3. Accumulation of osmoprotectants (μmol/g dry weight) in *E. coli* K-12 strains grown anaerobically at high osmolarity[a]

strain, medium additions	betaine	carnitine	proline	amino acids	trehalose
WT					
none	0[b]	0	255 ± 50	250 ± 60	0
betaine	190 ± 29	0	0	35 ± 6	0
carnitine	0	185 ± 17	0	30 ± 5	0
betaine and carnitine	220 ± 28	33 ± 6	0	nd[c]	0
proline	0	0	247 ± 25	nd	0
WG244					
none	0	0	245 ± 65	250 ± 60	0
betaine	194 ± 25	0	0	30 ± 5	0
carnitine	0	146 ± 44	0	40 ± 7	0
betaine and carnitine	199 ± 21	trace	0	nd	0
proline	0	0	216 ± 11	nd	0
CLG85					
none	0	0	0	20 ± 4	0
betaine	0	0	0	20 ± 4	0
carnitine	0	48 ± 14	0	20 ± 4	0
betaine and carnitine	0	60 ± 20	0	nd	0
proline	0	0	0	nd	0

[a] Cultures were grown at 37°C in MM plus 50 mM glycerol, 18 mM sodium fumarate and 0.1 % (wt/vol) casamino acids plus 0.25 M NaCl and 1 mM betaine, 1 mM D,L-carnitine, 1 mM betaine and 1 mM D,L-carnitine or 1 mM L-proline where indicated until late-exponential growth phase. Cells were washed, cytoplasmic solutes were extracted and quantified by HPLC. Values are the mean of triplicate samples of independent cultures.

[b] 0 indicates below detection limit; [c] nd, not determined

into the medium to allow for growth of CLG85. None of the three strains accumulated betaine, proline or carnitine in this low-osmolarity medium. Amino acid levels in these cells were 20-25 μmol g dry weight^{-1}, whereas no trehalose was detected (data not shown). The NaCl concentration applied to assess the response of anaerobically grown *E. coli* K-12 to osmotic stress in this medium was 0.25 M (Table 3). WT and WG244 both accumulated high levels of amino acids (250 ± 60 μmol g dry weight^{-1}), most likely due to the presence of casamino acids in the external medium, in contrast to CLG85. These amino acid pools in WT and WG244 consisted almost completely of L-proline (Table 3), which might explain the low levels found in CLG85 which is defective in all proline porters. Extra addition of 1 mM proline to the high-osmolarity anaerobic medium did not result in a further increase in intracellular proline levels, 247 ± 25 μmol g dry weight^{-1} and 216 ± 11 μmol g dry weight^{-1} in WT and WG244 were found, respectively (Table 3). The addition of 1 mM betaine to the high osmolarity medium resulted in comparable betaine levels in WT and WG244 (190 ± 29 μmol g dry weight^{-1} and 194 ± 25 μmol g dry weight^{-1}, respectively); no betaine could be detected in CLG85 (Table 3). When present in the external medium, carnitine accumulation was observed for all three strains, levels of 185 ± 17, 146 ± 44 and 48 ± 14 μmol g dry weight^{-1} were detected for WT, WG244 and CLG85, respectively. These results suggest that CaiT may have a role in the transport of carnitine in *E. coli* K-12 to alleviate osmotic stress under anaerobic conditions in the absence of ProP and ProU. When betaine and DL-carnitine were supplied together to the high osmolarity anaerobic medium, betaine was preferentially accumulated in both WT and WG244, in CLG85 only carnitine was accumulated. Moreover, carnitine is preferred to L-proline in WT and WG244 since intracellular proline was not detected in high osmolarity medium containg casamino acids and 1 mM DL-carnitine. Trehalose could not be detected in the anaerobically grown *E. coli* strains (Table 3). Accumulation of trehalose was also not seen in WT and WG244 grown anaerobically in MM containing glucose and NaCl (not shown). These results indicate repression of trehalose synthesis by anaerobiosis.

L-carnitine transport in *E. coli* K-12 strains. The entry of L-[^{14}C]carnitine into *E. coli* K-12 through the ProP and ProU transport systems was analyzed. The constitutive uptake of L-carnitine via ProP was elicited in an assay medium lacking NaCl using cells grown in the absence of NaCl, osmotic activation of ProP was achieved introducing 0.5 M NaCl in the uptake assay medium. ProU mediated carnitine transport was educible from L-[^{14}C]carnitine uptake in the presence of 0.5 M NaCl with cells grow at high osmolarity. Uptake of L-carnitine in the absence of NaCl was 1.2 ± 0.2 nmol min^{-1} mg protein^{-1} which increased to 3.2 nmol min^{-1} mg protein^{-1} when uptake was measured in the presence of 0.5 M NaCl (Table 4). As expected, L-[^{14}C]carnitine uptake was absent in WG244 and CLG85 which are both defective in ProP. When both ProP and ProU were functional, L-[^{14}C]carnitine was taken up with a rate of 11.2 ± 1.1 nmol min^{-1} mg protein^{-1} in WT. Uptake of L-[^{14}C]carnitine via ProU in WG244 proceeded at a rate of 1.9 ± 0.3 nmol min^{-1} mg protein^{-1} and again no uptake was seen in CLG85 that lacks the ProP and ProU systems (Table 4). ProP and ProU both appeared to have a rather low affinity for L-carnitine (K_m is

approximately 200 -250 μM; data not shown).

For comparison, uptake of L-[^{14}C]proline in the three *E. coli* K-12 strains was determined under the conditions used for the L-[^{14}C]carnitine uptake studies. When WT was grown without NaCl, the uptake rate of L-[^{14}C]proline in the absence of NaCl amounted to 11.2 $\pm$ 1.4 nmol min^{-1} mg protein^{-1} (Table 4). The addition of NaCl to the transport assay resulted in a proline uptake rate of 26.8 $\pm$ 2.7 nmol min^{-1} mg protein^{-1}. With PutP, ProP and ProU in operation, L-[^{14}C]proline was transported at a rate of 47.3 $\pm$ 4.5 nmol min^{-1} mg protein^{-1} in WT, the rate of L-[^{14}C]proline uptake via ProU in WG244 was 10.1 $\pm$ 1.6 nmol min^{-1} mg protein^{-1}. The uptake rates measured for L-proline agree with those previously reported for other *E. coli* K-12 mutant strains (18).

Table 4. Uptake of L-[^{14}C]carnitine and L-[^{14}C]proline by *E. coli* K-12 strains under different osmotic stress conditions[a]

	L-carnitine uptake rate (nmol min^{-1} mg of protein^{-1})			**L-proline uptake rate** (nmol min^{-1} mg of protein^{-1})		
	experimental conditions[b]			experimental conditions		
	G:no add.	G:no add.	G:NaCl	G:no add.	G:no add.	G:NaCl
strain	A:no add.	A:NaCl	A:NaCl	A:no add.	A:NaCl	A:NaCl
WT	1.2 $\pm$ 0.2	3.2 $\pm$ 0.3	11.2 $\pm$ 1.1	11.2 $\pm$ 1.4	26.8 $\pm$ 2.7	47.3 $\pm$ 4.5
WG244	nil	nil	1.9 $\pm$ 0.3	nil	nil	10.1 $\pm$ 1.6
CLG85	nil	nil	nil	nil	nil	nil

[a] The initial rates of L-[^{14}C]carnitine uptake (final concentration, 400 μM) and L-[^{14}C]proline (final concentration, 200 μM) were measured at 37°C in aerobically grown cells in MM containing 10 mM glucose in the absence or presence of 0.5 M NaCl. Uptake rates were determined over the first 10 min in glucose-energized cells in 50 mM potassium phosphate (pH 6.9) containing 5 mM $MgSO_4$ and 50 μg chloramphenicol per ml with or without 0.5M NaCl in the assay buffer.

[b] G, growth condition; A, assay condition; no add, no additons, NaCl, 0.5 M NaCl.

Carnitine transport in WT *E. coli* K-12 was studied in further detail by introducing unlabelled betaine, proline and ectoine into the assay mixture at 1 and 10 times the concentration of L-[^{14}C]carnitine (Fig. 4). With only ProP operational in WT (grown without NaCl, L-carnitine uptake assayed at high osmolarity), L-[^{14}C]carnitine transport was significantly affected by proline, betaine and ectoine. L-carnitine uptake was more severely inhibited in WT cells that were already exposed to osmotic stress in the growth medium (*i.e.* both ProP and ProU are functional). These results clearly indicate that L-carnitine transport in *E. coli* K-12 is mediated by ProP and ProU and that the ProU system seems to be the main transport system.

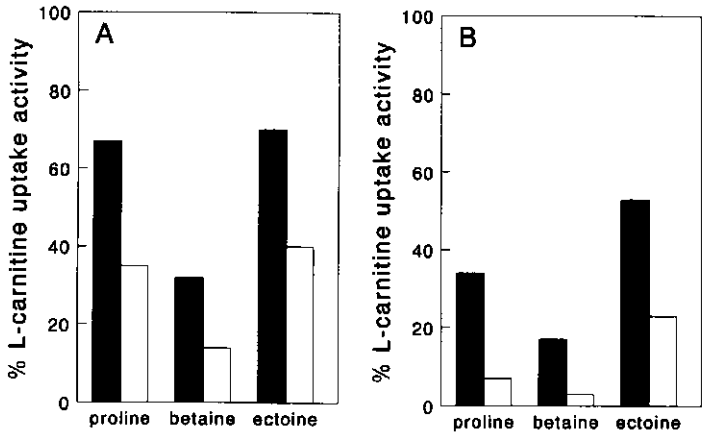

Figure 4. Uptake of L-[^{14}C]carnitine in WT *E. coli* K-12 in the presence of 1- and 10-fold excess unlabelled proline, betaine or ectoine. Uptake of L-[^{14}C]carnitine (final concentration, 400 μM) was determined in the presence of simultaneously added unlabelled analogs in the presence of 0.5 M NaCl. Cells were grown in MM plus 10 mM glucose the absence (panel A) or in the presence of 0.5 M NaCl (panel B). Results are presented as percentage activity of the control uptake in the absence of the competitor. Closed bars represent L-[^{14}C]carnitine uptake activity with the unlabelled analog present at the same concentration as L-[^{14}C]carnitine, open bars represent the uptake activity with the unlabelled analog present in 10-fold excess. The absolute rates of L-[^{14}C]carnitine transport in WT were 3.2 $\pm$ 0.3 nmol min^{-1} mg protein^{-1} and 11.2 $\pm$ 1.1 nmol min^{-1} mg protein^{-1} for cells grown at low osmolarity and high osmolarity, respectively.

L-[^{14}C]carnitine uptake rates in anaerobically grown WT were not significantly different from the values obtained in cells grown aerobically, whereas L-carnitine uptake in WG244 was slightly reduced under these conditions (data not shown). Possible uptake of L-[^{14}C]carnitine (final concentrations, 400 μM or 1 mM) via CaiT in CLG85 was analyzed in cells grown anaerobically in MM containing 50 mM glycerol, 18 mM sodium fumarate and 0.1% (wt/vol) casamino acids in the absence and presence of 1 mM L-carnitine with or without 0.25 M NaCl. Uptake of L-carnitine by CLG85 could, however, not be detected in the four sets of cells, neither in a low-osmotic assay mixture nor in a high-osmotic assay mixture (not shown).

Discussion

L-carnitine has been found recently to be a solute associated with osmotic adaptation in the gram-positive bacteria *L. monocytogenes* and *L. plantarum* (3, 23). *L. monocytogenes* has been shown to possess highly specific and separate transport systems for the osmolytes betaine and L-carnitine. Betaine transport proceeds via a proton-motive force dependent carrier protein and L-carnitine is taken up via an ATP-dependent transporter (35, 36). In *L. plantarum*, betaine and L-carnitine enter the cytoplasm via the same transport system (15). The data presented here show that carnitine accumulation allows the gram-negative bacterium

E. coli to grow at high osmolarity by uptake from the external medium both under aerobic and anaerobic growth conditions. Moreover, this paper sheds light on the role of carnitine in the possible mechanism of osmotic adaptation in *E. coli* O157:H7 during growth on low a_w meat products like salami and other fermented sausages. Due to the physiological role of carnitine in eukaryotic fatty acid metabolism, this compound is always abundant in meat products. This finding is important for the microbiological safety of meat products and has a large impact on the understanding of the pitfalls of food preservation.

Using *E. coli* K-12 strains carrying deletions in the *proP* and *proU* loci, the involvement of both ProP and ProU was demonstrated. Both in aerobically and anaerobically grown cells, the osmoprotective effect of carnitine in the WT strain was almost as prominent in the strain impaired in ProP (*i.e.* WG244) and the carnitine accumulation levels in WT and WG244 did not differ significantly, which suggest that the ProU system represents the main route for carnitine uptake in *E. coli* K-12 during osmotic stress. Carnitine failed to appear in the cytoplasm of the strain lacking PutP, ProP and ProU (*i.e.* CLG85) during aerobic growth at high osmolarity in the presence of exogenous carnitine, which rules out the participation of other transporters in the uptake of carnitine under these conditions.

Uptake of L-carnitine in WT via ProP was reduced to the same extent by L-proline and ectoine, whereas betaine showed the greatest inhibition. Indeed, ProP has a comparable affinity for L-proline, ectoine and L-carnitine (300 μM, 220 μM and 200-250 μM, respectively), which is significantly lower than that for betaine (K_m 44 μM) (7, 20). With both ProP and ProU operative in WT, L-carnitine uptake was severely decreased in the presence of the competitors tested with the highest inhibition observed with betaine. At onefold excess of betaine, only 17% of the L-[^{14}C]carnitine uptake activity remained, and a tenfold excess allowed only 3% of the original L-carnitine uptake activity. This can possibly be attributed to the high affinity of the ProU porter for betaine (K_m 1.4 μM).

The carnitine accumulation levels found in CLG85 grown anaerobically in glycerol/fumarate growing cells most probably result from transport via CaiT. However, it is unlikely that CaiT plays a role *in vivo* in osmoregulation in *E. coli* K-12 since anaerobic growth of CLG85 was not stimulated by exogenous carnitine under osmotic stress. Additionly, after anaerobic growth in low- and high-osmolarity glycerol/fumarate media supplemented with L-carnitine, this strain showed no measurable L-carnitine uptake. These results indicate a low expression level of CaiT and/or a very low uptake rate of L-carnitine via CaiT, insufficient to confer osmotolerance to *E. coli*. Carnitine concentrations in WT and WG244 grown anaerobically in the glycerol/fumarate MM containing 0.25 M NaCl plus 1 mM carnitine were comparable with those grown anaerobically in the glucose MM plus 0.25 M NaCl and 1 mM carnitine. Neither under the former nor under the latter growth condition, τ-butyrobetaine, the endproduct of the carnitine pathway, was detectable (data not shown). These results exclude a significant expression of the *cai* operon in *E. coli* K-12 at high osmolarity. Indeed, Jung *et al.* (21) previously observed that in *E. coli* O44 K74, synthesis of the enzymes carnitine dehydratase and crotonobetaine reductase was repressed after anaerobic growth on glycerol/fumarate in the presence of 0.65 M NaCl.

Most physiological studies on osmotic stress and osmoprotectant accumulation are

performed with cells grown aerobically, little is known about the role of osmoprotectants during anaerobiosis. The finding that the growth inhibition of *E. coli* K-12 at high osmolarity could not only be reversed by betaine and carnitine in cells grown aerobically, but additionly in anaerobically grown cells, seems of considerable importance in view of the occurrence of *E. coli* in the gastrointestinal tract where anaerobic conditions prevail. Moreover, it is of relevance for foods wich are packaged under vacuum or under a modified atmosphere. Betaine and carnitine are found in many foods (4, 31) and consequently these compounds will also be present in the human intestine. The results reported here suggest that *E. coli* is more sensitive to osmotic stress in the absence of oxygen, which could suggest that the bioenergetic pathway is the limiting step for growth under osmotic stress. The accumulation levels of betaine and carnitine found in the present study in anaerobically grown cells are two- to threefold lower than those found in aerobically grown cells which is most likely attributable to the different NaCl concentrations used, 0.5 M NaCl and 0.25 M NaCl in aerobically and anaerobically grown cells, respectively.

In this study, we assessed that carnitine can function as an osmoprotectant for *E. coli*, including the enterohaemorrhagic *E. coli* O157:H7, and that this compound enters via ProP and ProU in aerobically and anaerobically osmotically stressed *E. coli*. Moreover, it was demonstrated that no significant uptake of carnitine in *E. coli* K-12 takes place via CaiT to alleviate osmotic stress. It has been demonstrated that *E. coli* is also able to import a number of other compounds with osmoprotective effects via ProP and ProU next to betaine and L-proline. Examples include taurine (28), ectoine (20), choline (24), pipecolic acid, *N*-dimetylglycine, dimethylsulfoniopropionate (16) and several betaines like γ-butyrobetaine, alanine betaine, proline betaine and pipecolate betaine (19, 29). Therefore, the ProP and ProU systems seem to be rather unspecific and are most likely the main osmoporters involved in osmolyte influx into *E. coli*, thereby allowing growth in a wide variety of environments of low a_w.

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8

Modifications of membrane phospholipid composition in nisin-resistant *Listeria monocytogenes* Scott A

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Abstract

A nisin-resistant variant (NIS^R) of *Listeria monocytogenes* Scott A was isolated by stepwise exposure to increasing concentrations of nisin in brain heart infusion (BHI) broth. The NIS^R strain was about 12 times more resistant to nisin than the wild-type (WT) strain. Accordingly, higher nisin concentrations were required to dissipate both components of the proton motive force in the NIS^R strain as compared to the WT strain. Comparison of the membrane fatty acyl composition of the sensitive strain with that of its NIS^R derivative revealed no significant differences. From phospholipid headgroup composition analysis and phospholipid biosynthesis measurements during growth in the absence and presence of nisin, it could be inferred that the NIS^R strain produces relatively more phosphatidylglycerol (PG) and less diphosphatidylglycerol (DPG) in comparison with the parent strain. Monolayer studies using pure lipid extracts from both strains showed that nisin interacted more efficiently with lipids derived from the WT strain than those of the NIS^R strain, reflecting qualitative differences in nisin sensitivity. Involvement of the cell wall in acquisition of nisin resistance was excluded, since WT and NIS^R showed a comparable sensitivity to lysozyme. Recently, it has been demonstrated that nisin penetrates more deeply into lipid monolayers of DPG than those of other lipids including PG, phosphatidylcholine (PC), phosphatidylethanolamine (PE), monogalactosyldiacylglycerol and digalactosyldiacyl-glycerol (Demel, R.A., T. Peelen, R.J. Siezen, B. de Kruijff and O.P. Kuipers. 1996. Eur. J. Biochem. **235**:267-274, 1996). Collectively, the mechanism of nisin-resistance in this *L. monocytogenes* NIS^R strain is attributed to a reduction in the DPG content of the cytoplasmic membrane.

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Introduction

Nisin is a 34-amino-acid polypeptide bacteriocin (lantibiotic) produced by *Lactococcus lactis* subsp. *lactis*. Since its discovery in the 1920s, nisin has proven to be an effective inhibitor of a broad spectrum of Gram-positive bacteria, including the foodborne pathogen *Listeria monocytogenes*. It is the only bacteriocin produced commercially with a legal status to be used as a food additive (10). Two natural variants of nisin are known: nisin A and nisin Z, which are equally distributed among nisin-producing *L. lactis* strains (14). Nisin A and nisin Z differ by a single substitution at position 27, being His in nisin A and Asn in nisin Z (38). The nisin molecule has an amphiphatic character, the N-terminal part of the nisin molecule contains a relatively high number of hydrophobic residues, whereas the C-terminal part is more hydrophilic. Nisin is cationic due to the presence of three lysine residues and one (in nisin Z) or two (in nisin A) histidine residues and the absence of glutamate and aspartate. The pK values of the side-chain groups in histidine and lysine residues are 6.5 and 10.0 respectively and the net charge of nisin is therefore pH dependent (24, 30).

The primary target of nisin in sensitive vegetative bacteria has been shown to be the energy-transducing cytoplasmic membrane. Addition of nisin to Gram-positive bacteria results in an efflux of essential small cytoplasmic components (amino acids, monovalent cations, ATP), depletion of both components of the proton-motive force ($\Delta\psi$ and ΔpH) and cessation of biosynthesis (1, 6). Nisin has been shown to act on several species of Gram-negative bacteria, provided that the integrity or barrier function of the outer membrane is first disrupted (25). Nisin does not require a specific protein receptor to exert its antimicrobial activity. Experiments with intact cells, cytoplasmic membrane vesicles and liposomes indicate that a *trans*-negative $\Delta\psi$ of sufficient magnitude (15) is necessary for the activity of nisin. Studies by Gao *et al.* (17) and García Garcerá *et al.* (18) however demonstrated that a ΔpH (inside alkaline) is dissipated by nisin in the absence of a $\Delta\psi$, suggesting that a ΔpH is as efficient as $\Delta\psi$ in promoting nisin action. In addition, the threshold $\Delta\psi$ is influenced by the nisin concentration and the phospholipid composition of the cytoplasmic membrane (15, 17, 18, 28).

Microorganisms are often able to overcome the inhibitory effect of antibiotics and preservatives. Emergence of nisin-resistant mutants, which are generated when nisin-sensitive cells are exposed to relatively high nisin concentrations, has been described for several *Lactobacillus* spp., *Streptococcus* spp., *Leuconostoc* spp., *Bacillus* spp., *Clostridium* spp., *Staphylococcus aureus* and *L. monocytogenes* (5, 7, 8, 36, 37). Harris *et al.* (20) detected mutant strains of *L. monocytogenes* at a frequency of 10^{-6} and 10^{-8} which were able grow at nisin concentrations 5-10 times higher than the original population, indicating the potential for nisin-resistant variants to arise from widespread application of the antibiotic. Insight into the mechanism of nisin resistance is important because it will provide a rational basis for its application in the food industry. Several mechanisms of nisin-resistance in Gram-positive bacteria can be envisaged. In view of the consensus that the bacterial membrane is the site of nisin action, the nisin-resistant phenotype may develop from alterations in the cell envelope (9, 32, 36, 37).

In the present paper, we investigate the mechanism of nisin resistance in *L. monocytogenes* by comparing both fatty acyl and headgroup compositions of membrane phospholipids in nisin-sensitive and nisin-resistant *L. monocytogenes* Scott A. Furthermore, the capability of both strains to synthesize phospholipids during exposure to nisin has been investigated. Monolayer studies with pure lipids extracts from both strains have been used to assess specific interactions of nisin with lipids in further detail. Combined, these approaches provide important new information on the role of membranes in nisin resistance.

Materials and Methods

Bacterial strains and culture conditions. The wild-type (WT) strain of *L. monocytogenes* Scott A and its nisin-resistant derivative (NIS^R) were grown in brain heart infusion (BHI) broth. Stock cultures were maintained in the same medium solidified with agar. The NIS^R strain was acquired by increasing stepwise the exposure to nisin A (0.6, 1.0, 1.6, 2.6, 3.4, 5.0, 7.0, 10, 15, 30 mg/l); the resistant strain is 10-15 times more resistant than the parent strain, but fails to grow in BHI broth containing 40 mg nisin per liter. Although the nisin resistance was stable for at least ten passages through nisin-free medium (24 h intervals), nisin was always incorporated into BHI agar at a concentration of 10 mg/l for routine sub-culture and maintenance of the mutant strain.

Cultures were grown in Erlenmeyer flasks containing 20% volume of medium at 7°C or 30°C in a gyrorotatory incubator (New Brunswick Scientific, Edison, NJ) at 150 rpm. Growth was followed by measuring turbidity at 600 nm (OD_{600}) or 620 nm or by plate counting on tryptic soya agar (TSA).

Determination of Minimal Inhibitory Concentrations (MICs). *L. monocytogenes* was grown overnight at 30°C in BHI, inoculated with approximately 10^5 cells per ml into the different wells of a microtitre plate which contained fresh BHI with increasing nisin concentrations. The MIC was determined as being the lowest nisin concentration which prevented growth after incubation for 24 hours at 30°C.

Measurement of intracellular pH and membrane potential in intact cells. The intracellular pH was determined using the fluorescent probe 5 (and 6-)carboxyfluorescein diacetate succinimidyl ester as described by Breeuwer *et al.* (4) in 50 mM potassium phosphate buffer, pH 7.2. Assays were performed in the presence of valinomycin (1 μM) in order to dissipate the membrane potential.

The $\Delta\psi$ (inside alkaline) was monitored by quenching the potential-sensitive fluorescent probe 3,3-dipropylthiadicarbocyanine ($DiSC_3[5]$) (excitation wavelength, 643 nm; emission wavelength, 666 nm). Membrane potential measurements were performed in 50 mM potassium *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (K-HEPES) buffer (pH 7.2) in the presence of nigericin (1 μM) to prevent the generation of a transmembrane pH gradient. Fluorescense measurements were determined using a Perkin-Elmer LS 50 spectrofluorimeter at 30°C with continuous stirring.

Isolation, fractionation and identification of lipids. *L. monocytogenes* cultures were grown in BHI until mid-exponential phase (OD_{600} = 0.7), sedimented by centrifugation, washed twice in 50 mM potassium phosphate buffer (pH 6.9) containing 5 mM $MgSO_4$ and the total lipids were extracted from cell pellets using the method of Bligh and Dyer (3) as detailed by Kates (26).

It has been suggested that Gram-positive bacteria should be pretreated with lysozyme for a full extraction of lipids, particularly diphosphatidylglycerol, from stationairy phase cultures (13, 16). However, these studies show that in log phase cultures, good extraction can be obtained without lysozyme, so no lysozyme treatment was used.

Individual lipids were separated from total lipid extracts by thin-layer chromatography (TLC).

Initially, phospholipids were tentatively identified by comparing their mobilities with those of standards in one- and two-dimensional TLC both on commercial precoated silica gel 60A plates (Whatman, Clifton, NJ) and homemade plates poured with silica gel 60H (Merck, Darmstadt, Germany) in the following solvent systems: chloroform - methanol - acetic acid -water (85:15:10:3.5, by volume); chloroform - methanol - water (65:25:4, by volume); chloroform - methanol - 28% ammonia (65:25:4, by volume); chloroform - methanol - 28% ammonia (65:35:5, by volume); chloroform - methanol - acetone - acetic acid - water (6:2:8:2:1, by volume) and chloroform - methanol - acetone - acetic acid - water (10:2:4:2:1, by volume). Individual lipids on TLC plates were visualized using the following stains: iodine vapour (general lipid detection), ninhydrin (amino groups), α-naphthol (sugar moieties), phosphate stain (phosphate groups) (26). Phospholipids were quantified by phosphorus analysis using the modified Bartlett (2) assay.

Fatty acid analysis. Aliquots of the phospholipid extract were used for the preparation of fatty acid methyl esters by transmethylation using concentrated 2.5% H_2SO_4 (vol/vol) in anhydrous methanol. The fatty acid methyl esters were extracted using petroleum ether (boiling point 60-80°C) and subsequently separated isothermally at 165°C by gas liquid-chromatography (GLC) using a Perkin-Elmer F33 gas chromatograph equipped with a glass column (1 m x 4 mm ID) packed with 5 % (wt/wt) SP2100 (Supelco, Bellefont, PA) as the stationary phase. Fatty acid methyl ester compositions were determined by measuring peak areas after identification using authentic standards and comparison of retention times.

Phospholipid radiolabelling and extraction. Cells were grown overnight, inoculated into 80 ml fresh medium and allowed to grow to an OD_{600} of about 0.2. The culture was divided into two 40 ml portions and were pulse-labelled at time zero with 52 μl [1-^{14}C]acetate (final concentration 9.62 kBq/ml, 4.8 μM) and immediately 2.0 mg/l nisin A (*i.e.* 80 μl of a sterile stock solution in 0.02 M HCl containing 1,000 mg of nisin A per l) was added to one flask and 80 μl 0.02 M HCl to the other flask which served as the control. Rates of [^{14}C]acetate incorporation into lipids were determined as follows: 3 ml samples of the labelled cultures were withdrawn at different time intervals and the lipids were extracted by rapidly mixing the culture sample with 11.25 ml methanol/chloroform (2:1, vol/vol). After at least 1 h, a further 3.75 ml of chloroform and 3.75 ml of water were added. Subsequently, the mixture was centrifuged briefly, the upper phase removed, and the chloroform layer washed with 14 ml methanol/water (10:9, vol/vol). The final washed chloroform extracts were evaporated to dryness under N_2, and the lipid residue redissolved in 400 μl of chloroform.

Radioactivity measurements. The radioactivity in total lipid extracts was determined by transferring 40 μl of each of the lipid extracts into plastic scintillation vials. The solvent was evaporated and 10 ml Ecolite (ICN, Cal, USA) was added as scintillant and the radioactivity was measured with a Wallac 1211 Rackbeta liquid scintillation counter (LKB, Biochrom, UK). To determine the radioactivity in individual lipids, the remainder of each lipid was separated by TLC on homemade plates poured with silica gel 60H (Merck, Darmstadt, Germany) using chloroform - methanol - acetic acid - water (85:15:10:3.5, by volume) as the solvent system. In order to aid the visualization of phospholipid spots, approximately 0.1 mg carrier lipid was added to the lipid samples before chromatography. The areas of individual spots were scraped into scintillation vials for determination of the radioactivity as above.

Lysozyme action. Early-stationairy phase cultures of WT and NIS^R were inoculated into fresh BHI (10% vol/vol) and growth was monitored at 30°C by measuring turbidity at 620 nm using a Novaspec II spectrometer (Pharmacia Biotech). At an optical density of 0.5-0.6, either 2 mg/ml or 5 mg/ml egg white lysozyme was added to the cultures and the optical density was recorded every 30 min for 4

h. In addition, after 1 and 3 h, appropriate serial dilutions of the samples were plated on TSA. Plates were incubated 2-3 days at 30°C.

Monolayer experiments. The Wilhelmy plate method was used to measure nisin-induced changes in the surface pressure of purified bacterial total lipid extract. Purified lipid was obtained via conversion of the anionic lipids to their sodium salts and the removal of proteins from the lipid extract by silica column chromatography (27, 43). Analysis by TLC showed that the lipid composition was not affected upon purification. Monolayers were formed by spreading a lipid containing chloroform solution on the air/buffer (10 mM Tris, pH 7.4) interface to an initial surface pressure between 18 and 40 mN/m. Nisin Z was added at a saturating concentration (1.2 μg/ml) through a small injection hole to the subphase which was stirred continuously. The time-dependent surface pressure increase after the addition of nisin was measured until a stable surface pressure was reached. The experiments were performed at room temperature.

Protein determination. Protein concentrations were determined by the method of Lowry *et al.* (31) using bovine serum albumin as a standard.

Statistical evaluation. Statistical analysis of data was performed with a two-tailed Student *t*-test for unpaired observations. Differences were considered significant if $p < 0.05$. Values are expressed as the mean $\pm$ standard deviations.

Chemicals. Sodium[1-^{14}C]acetate (2.0 GBq mmol^{-1}) was purchased from Amersham (Buckinghamshire, UK). BHI and TSA were from Difco Laboratories (Detroit, MI, USA). Nisin A was obtained as nisaplin from Sigma (St. Louis, Mo., USA) which contains 2.5% nisin (wt/wt). Nisin Z was produced and purified as previously described (38). Fluorescent dyes were obtained from Molecular Probes Europe B.V. (Leiden, The Netherlands). All other chemicals were reagent grade and obtained from commercial sources.

Results

Effect of nisin on bioenergetic parameters in WT and NIS^R *L. monocytogenes* Scott A. Nisin MIC values for both *L. monocytogenes* strains were determined in BHI broth. The WT strain had a MIC of 3.2 $\pm$ 0.2 mg/l compared with 37.5 $\pm$ 2.5 mg/l for the NIS^R strain. The influence of nisin on the ΔpH and $\Delta\psi$ was determined at an external pH of 7.2, which is the pH of BHI broth. Upon addition of 1.3 mg of nisin per l, an immediate dissipation of the ΔpH was observed in WT *L. monocytogenes* Scott A (Fig. 1). This nisin concentration had no effect on ΔpH in NIS^R. Titration with nisin resulted in a gradual decrease of the intracellular pH in the latter strain and a complete collapse of the ΔpH was observed after addition of in total 11.7 mg of nisin per l (Fig. 1). The $\Delta\psi$ in WT collapsed after addition of 0.25 mg of nisin per l, whereas the $\Delta\psi$ of the NIS^R strain was only slightly affected by this nisin concentration, requiring a final nisin concentration of 0.5 mg/l for complete collapse of the $\Delta\psi$ (Fig. 2). Valinomycin completely abolished the $\Delta\psi$ in both strains. These results demonstrate that nisin susceptibility in BHI broth correlates with the effect of nisin on bioenergetic parameters in *L. monocytogenes* Scott A.

Membrane composition of wild-type (WT) and nisin-resistant (NIS^R) *L. monocytogenes* grown at 30°C and 7°C. When nisin is used as a biopreservative in minimally processed ready-to-eat refrigerated foods, the psychrotroph *L. monocytogenes* is exposed to nisin at low

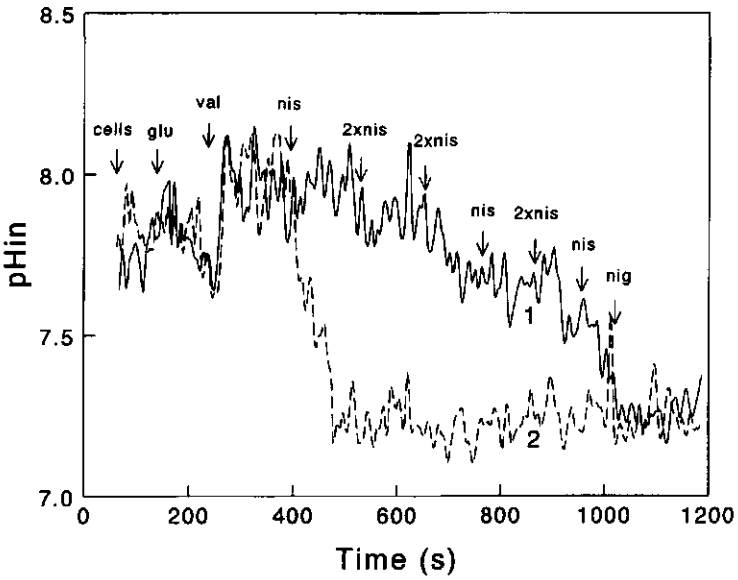

Figure 1. Effect of nisin on the intracellular pH of glucose-energized cells of WT and NIS^R *L. monocytogenes* Scott A. Harvested cells were washed and resuspended in 50 mM potassium *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (K-HEPES) buffer (pH 8.0) and incubated for 10 min at 30°C in the presence of 1 μM 5 (and 6-)carboxyfluorescein diacetate succinimidyl ester. Cells were washed in 50 mM potassium phosphate buffer (pH 7.2) and the nonconjugated fluorescent probe was eliminated by incubation of the cells in the presence of 0.5% (wt/vol) glucose at 30°C followed by washing of the cells in 50 mM potassium phosphate buffer (pH 7.2). At the times indicated, cells (final OD_{620}, 0.067), 0.5 % (wt/vol) glucose (glu), 1 μM valinomycin (val), 1.3 mg/l nisin (nis) or 2.6 mg/l nisin (2xnis), and 1 μM nigericin (nig) were added to the phosphate buffer. Assays were performed with NIS^R (trace 1) and WT (trace 2).

temperature. Membrane fatty acid and phospholipid headgroup composition of mutant and parent strain were therefore determined in cells grown in BHI broth both at high (30°C) and low (7°C) temperature. The primary response of fatty acid composition in both WT and NIS^R *L. monocytogenes* Scott A to a decrease in growth temperature appeared to be a marked increase in *anteiso*-C15:0 at the expense of *anteiso*-C17:0 (Fig. 3), which is in agreement with reports in the literature (39, 40). For a given temperature, only very small differences between the fatty acid compositions of the WT and the NIS^R strains were detected: the resistant mutant contained more C16:0, C18:0 and C18:1 and less of the branched chain fatty acids *anteiso*-C15:0 and *anteiso*-C17:0 compared to the wild type strain at both temperatures (Fig. 3). However, statistical analysis indicated that these differences in fatty acid composition were not significant at the 95% confidence level.

The major phospholipids in *L. monocytogenes* Scott A were identified as being phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) and a phospholipid that stained positively with α-naphthol, *i.e.* a phosphoglycolipid (PGL) (Table 1). The remaining phospholipids were minor components and no further attempt was made to identify them. Data from previous studies indicate the presence of PG, DPG and a PGL, but give no firm description of the minor phospholipids in *L. monocytogenes*; possible identities include phosphatidylserine, (amino)phosphoglycolipids, phosphatidylinositol and bisphosphatidylglycerolphosphate (13, 29, 34, 37, 39). Statistical analysis of the phospholipid content of either WT or NIS^R grown at 30°C and 7°C revealed no temperature-dependent differences for either strain. In comparison, there was a significant increase in phosphatidylglycerol (PG) and a decrease in the other phospholipids in NIS^R compared to WT at both growth temperatures (Table 1).

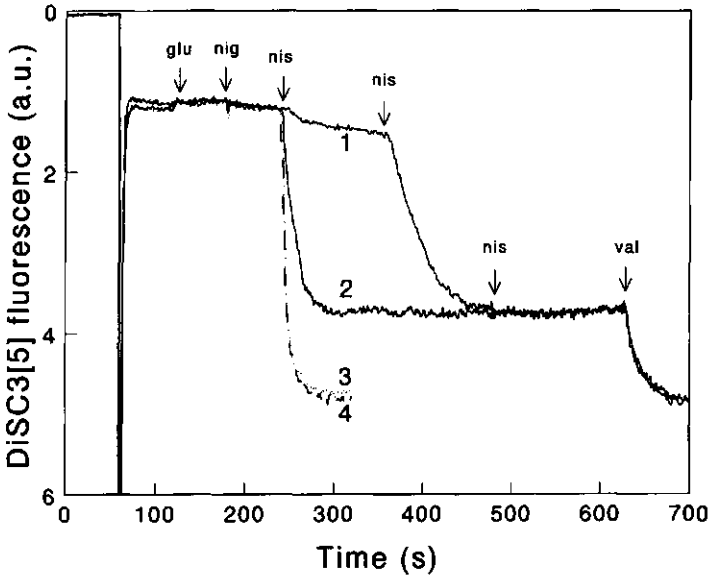

Figure 2. Effect of nisin on the membrane potential of glucose-energized cells of WT and NIS^R *L. monocytogenes* Scott A. Cells were grown in BHI, washed and resuspended in 50 mM K-HEPES buffer (pH 7.2). Cell suspensions of NIS^R (trace 1) and WT (trace 2) were diluted to an OD_{620} of 0.25 and 3,3-dipropylthiadicarbocyanine ($DiSC_3[5]$) was added to a final concentration of 5 μM. The following additions were made at the times indicated by the arrows, 0.5 % (wt/vol) glucose (glu), 1 μM nigericin (nig), 0.25 mg/l nisin (nis), and 1 μM valinomycin (val) were added. The dashed lines show the results of seperate experiments with NIS^R (trace 3) and WT (trace 4) in which valinomycin at a concentration of 1 μM was added.

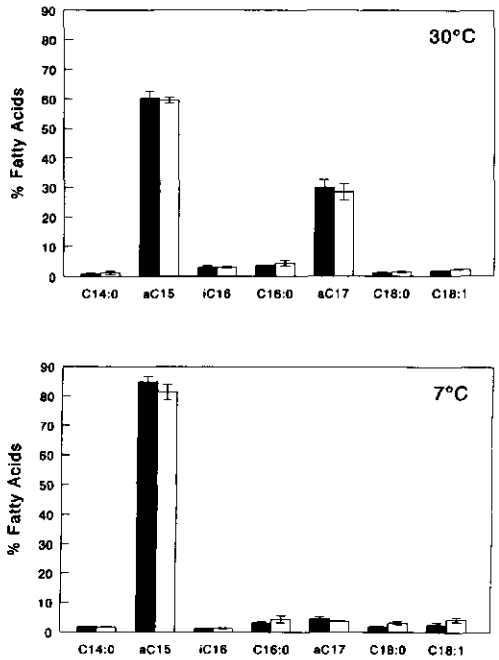

Figure 3. Fatty acid compositions (wt%) of parental (WT) (*black bars*) and resistant (NIS^R) (*white bars*) strains of *L. monocytogenes* Scott A. Values are the means ± standard deviation of analysis in duplicate of lipids extracted from three separate cultures grown in BHI at 30°C (upper graph) or at 7°C (lower graph) until late-exponential growth phase; a = *anteiso*-branched; i = *iso*-branched.

Table 1. Phospholipid composition (wt% phosphorus content) of wild-type (WT) and nisin resistant (NIS^R) *L. monocytogenes* Scott A[a)]

	WT		NIS^R	
Phospholipid[b)]	30°C	7°C	30°C	7°C
PG	62.3 ± 0.54[c)]	62.1 ± 0.57	68.0 ± 0.64	69.1 ± 0.27
PGL	9.4 ± 0.06	9.4 ± 0.13	8.8 ± 0.05	8.9 ± 0.18
DPG	12.7 ± 0.05	12.8 ± 0.31	9.6 ± 0.12	9.8 ± 0.05
others[d)]	15.6 ± 0.25	15.7 ± 0.39	13.6 ± 0.60	12.2 ± 0.29

[a)] Cultures were grown in BHI with shaking at 30°C or 7°C until mid-exponential phase (OD_{600}=0.7).
[b)] Abbreviations: PG, phosphatidylglycerol; PGL, phosphoglycolipid; DPG, diphosphatidylglycerol.
[c)] Values are the mean of triplicate samples of independent cultures ± standard deviation.
[d)] Represent several minor phospholipids and may include phosphatidylserine, (amino)phosphoglycolipids, phosphatidylinositol and bisphosphatidylglycerolphosphate.

Phospholipid biosynthesis in WT and NIS^R *L. monocytogenes* Scott A during growth in the absence and presence of nisin. A radioactive precursor of lipid biosynthesis, [^{14}C]acetate, was used to quantitate the lipid biosynthesis in the two strains during exposure to nisin. Nisin was added to an exponentially-growing culture together with the label at time zero. It was established that a nisin concentration of 2.0 mg/l was the optimal concentration for the pulse-labelling experiments, since this concentration was enough to inhibit growth of the WT strain, but did not cause cell lysis. In the control experiment (no nisin present), the culture continued growing exponentially, whereas in the presence of nisin, growth stopped and resumed after a lag period of approximately 140 min (Fig. 4A). During this period the viable cell counts remained constant (data not shown). The incorporation of [^{14}C]acetate into the lipids of WT in BHI occurred at a rate that was roughly twice that of WT growing in BHI plus nisin (Fig. 4A). The cells that continued growing 140 min after nisin addition were subcultured to determine their MIC for nisin, which indicated that these cells had not developed resistance during this period (data not shown).

The rate of lipid biosynthesis in NIS^R growing in BHI in the absence of nisin was comparable with that of WT growing under the same conditions. Growth of NIS^R in the presence of nisin was indistinguishable from that in the absence of nisin. Accordingly, the addition of nisin to the culture medium did not affect the rate of [^{14}C]acetate incorporation into the lipids of NIS^R (Fig. 4B).

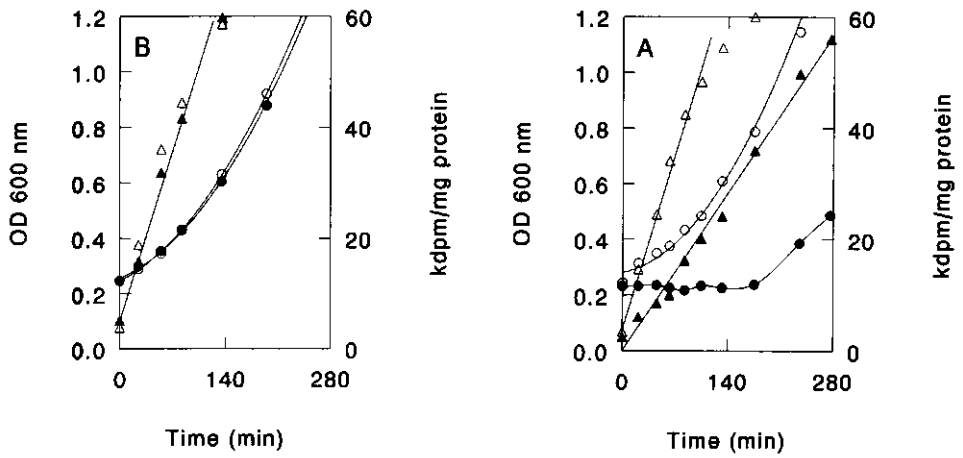

Figure 4. Incorporation of [^{14}C]acetate in phospholipids of WT (A) and NIS^R (B) *L. monocytogenes* Scott A during growth at 30°C in the absence or presence of nisin. The radioactive precursor [^{14}C]acetate (9.6 kBq/ml, 4.8 μM) was added to cultures growing exponentially in BHI together with nisin (2 mg/l) at time zero if required. Samples were removed for lipid extraction and subsequent determination of ^{14}C-incorporation (*triangles*). Growth was monitored as OD_{600} (*circles*). *Open* and *closed symbols* represent the results in the absence and presence of nisin, respectively.

Biosynthesis of the major individual phospholipids PG and DPG in WT and NIS^R *L. monocytogenes* Scott A during nisin exposure. The biosynthesis of PG and DPG, as measured by the incorporation rates of [^{14}C]acetate, during growth of WT in the absence of nisin were higher than those of cultures growing in the presence of nisin (Fig. 5A and 5B), which is consistent with the effect of nisin on total lipid biosynthesis as shown in Fig 4A. Furthermore, the amount of radioactivity incorporated in DPG relative to PG (*i.e.* the DPG/PG ratio) remained more or less unaffected upon nisin addition. This result implies that nisin does not directly affect phospholipid biosynthesis in WT *L. monocytogenes*. The incorporation rates of [^{14}C]acetate in both PG and DPG of NIS^R were similar with or without nisin (Fig. 5C and 5D). However, the nisR strain incorporates more [^{14}C]acetate in PG and less in DPG compared to the WT strain, which indicates that in NIS^R more PG and less DPG is produced.

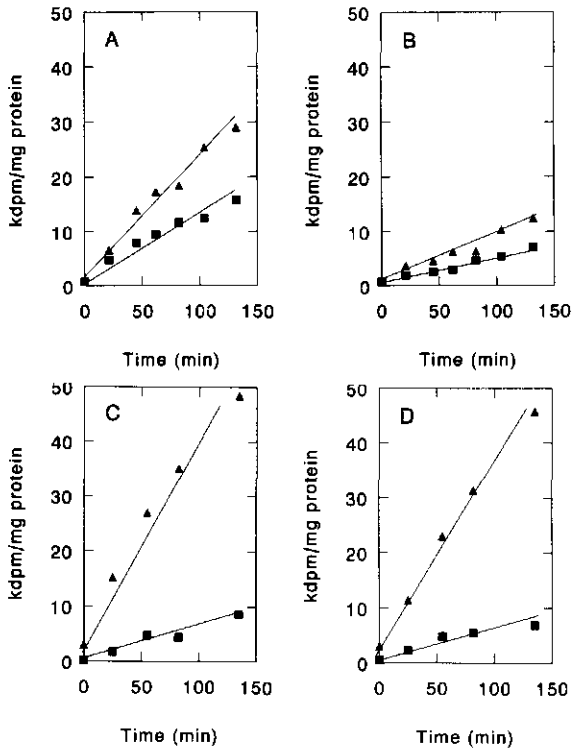

Figure 5. Effect of nisin on PG and DPG synthesis in WT and NIS^R *L. monocytogenes*. *L. monocytogenes* Scott A cultures growing exponentially at 30°C were incubated with [^{14}C]acetate (9.6 kBq/ml, 4.8 μM) and at intervals samples were extracted, the individual phospholipids were fractionated by TLC, and the incorporation of [^{14}C]acetate into phosphatidylglycerol (▲) and diphosphatidylglycerol (■) was measured. Panels A and B represent rates of incorporation in the WT strain in the absence and presence of nisin (2.0 mg/l), respectively, while panels C and D, show incorporation rates in the NIS^R strain in the absence and presence respectively of nisin.

Sensitivity of WT and NIS^R *L. monocytogenes* Scott A to lysozyme. Recently, it has been demonstrated that nisin-resistance is based on cell wall changes in some *Listeria* strains (9, 32). Lysozyme addition (final concentration, 2 mg/ml) during exponential growth of WT and NIS^R in BHI, resulted in growth inhibition of both strains. At this lysozyme concentration, both WT and NIS^R numbers decreased by ca. 0.5 log units over 3h. The effect of 5 mg/ml lysozyme on log phase cultures was a higher viability loss for WT and NIS^R, a reduction of ca. 1 log unit was recorded for both strains (data not shown). These results indicate that NIS^R and WT have a comparable sensitivity towards lysozyme.

Interaction between nisin Z and lipid monolayers of WT and NIS^R *L. monocytogenes*. Monolayers of pure lipids from WT and NIS^R *L. monocytogenes* were formed to determine specific interactions of nisin with the lipids. For these experiments, purified nisin Z was used instead of nisin A. The MIC values for both nisin A and nisin Z against WT and NIS^R were the same (data not shown). The changes in surface pressure after the addition of nisin to the aqueous subphase are interpreted as being a result of the insertion of nisin into the lipid phase. In the absence of a lipid monolayer, nisin maximally increases the surface pressure up to 16 mN/m at the concentration used (data not shown). This indicates that at an initial surface pressure above 16 mN/m, the surface pressure increases after the addition of nisin to the subphase of the lipid monolayer, are a direct consequence of nisin-lipid interactions. The surface pressure increase is less at higher initial surface pressures due to the tighter lipid packing of the monolayer; surface pressures between 30 and 35 mN/m are believed to be relevant for biological membranes (12).

Monolayers of WT lipid extracts showed large pressure increases after the addition of nisin (Fig. 6). The pressure changes induced by nisin in monolayers of NIS^R lipid were significantly lower. The results show that nisin interacts more efficiently with the lipids of WT membranes than those of NIS^R, which mirrors the difference in nisin sensitivity of the bacterial strains. Considering the effect of external pH on nisin efficiency, monolayers were also formed with lipids from both strains on a subphase of 10 mM Tris buffer pH 6.0 and the effect of nisin addition was evaluated and compared to results obtained with a subphase of 10 mM Tris buffer pH 7.4. Pressure changes at pH 6.0 were lower than at pH 7.4; at an initial surface pressure of 22 mN/m, the pressure change was reduced by 15-20% for both strains and at an initial surface pressure of 31 mN/m, this reduction was 30-40% (data not shown). These results suggest that a lower pH reduces the nisin-lipid interaction in monolayer studies. Divalent cations (*i.e.* Mg^{2+} and Ca^{2+}) have been shown to have an inhibitory effect on the action of nisin Z against *L. monocytogenes*, which has been interpreted as a neutralization of the negatively-charged phospholipid headgroups (1). The effect of divalent cations on nisin-lipid interactions was tested by adding 10 mM $MgSO_4$ to the subphase of lipid monolayers of WT and NIS^R lipids. The pressure increases recorded with monolayers after nisin addition to a subphase containing $MgSO_4$, at initial pressures of 32 mN/m, were reduced to approximately 50% compared to the low-ionic-strength condition for both strains (data not shown). For comparison, the influence of Mg^{2+} ions on nisin MIC values in BHI broth was determined. The MIC value for WT (3.2 $\pm$ 0.2 mg/l) rose with

increasing $MgSO_4$ concentration and reached a value of 4.0 ± 0.25 mg/l at a $MgSO_4$ concentration of 10 mM. For NIS^R this increase was from 37.5 ± 2.5 (no $MgSO_4$) to 50 ± 4 mg/l in the presence of 10 mM $MgSO_4$. In the presence of 250 mM $MgSO_4$, the nisin MIC value for WT strain was still 3 to 4 times lower (*i.e.* about 10 mg/l) than the value determined for NIS^R in the absence of $MgSO_4$ (data not shown), which indicates that very high concentrations of divalent cations must be applied to WT to reach the same resistance level as with NIS^R.

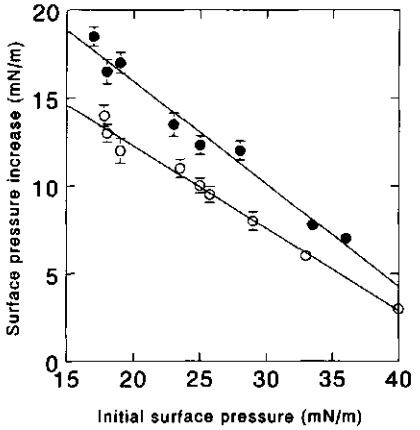

Figure 6. Effect of nisin on surface pressure of isolated lipids of WT and NIS^R *L. monocytogenes*. Surface pressure increases were determined after the injection of nisin Z underneath monolayers of purified total lipids extracted from WT (●) and NIS^R (○) strains of *L. monocytogenes* Scott A at different initial surface pressures. Nisin Z (41 μg) was injected in a subphase comprising 35 ml 10 mM Tris, pH 7.4.

Discussion

In the present paper, we showed that higher nisin concentrations were required to dissipate the ΔpH and $\Delta\psi$ in a nisin-resistant variant of *L. monocytogenes* Scott A (NIS^R) as compared to its parent wild-type (WT) strain, which correlates with the nisin susceptibility of the strains in BHI broth. This suggest a possible role for the energy-transducing cytoplasmic membrane in the acquisition of nisin-resistance in *L. monocytogenes* Scott A. The phenomenon of nisin resistance in *L. monocytogenes* Scott A was studied in detail with a focus on membrane composition.

Comparison of the fatty acid composition at one particular temperature of the NIS^R isolate of *L. monocytogenes* Scott A with that of the WT strain at both growh temperatures tested (7°C and 30°C), revealed no significant differences. Ming and Daeschel (37) found that their stable nisin-resistant mutant of *L. monocytogenes* contained a greater proportion of straight chain fatty acids, whereas the parent contained more branched-chain fatty acids; no changes in unsaturation of lipid acyl chains were reported. However, the specific growth rate of their resistant mutant was decreased, especially at low temperature, which could indicate a less flexible membrane, whereas our mutant exhibited the same growth characteristics as the

parent strain both at 7°C and 30°C (not shown). Furthermore, the proton-motive-force generated ($\Delta\psi$ and ΔpH) in the WT and the NIS^R strain is similar indicating that bioenergetics is not affected in the latter strain (Figs. 1 and 2).

The phospholipid compositions of the WT and NIS^R strains did not alter significantly with growth temperature, which agrees with data described for other bacteria (42). Significant changes in the proportional ratios of the phospholipids in WT and NIS^R *L. monocytogenes* were however found, the most prominent alteration in NIS^R compared to WT being the higher ratio between PG and DPG (approximately 7 compared to 5, Table 1). This observation was matched by the results of the phospholipid metabolism studies, which demonstrated that the incorporation rate of [^{14}C]acetate in DPG relative to PG is significantly reduced in the mutant strain (Fig. 5). A direct comparison of the DPG:PG ratios calculated from the mass values given in Table 1 with those obtained from the metabolism studies (Fig. 5) is not justified because the former are based on phosphorus content whereas the latter represent [^{14}C]acetate incorporation in the carbon skeleton; thus, differences in the rate of turnover of different parts of the molecule, particularly the fatty acyl chains, would distort the values obtained. The finding that NIS^R produces relatively more PG and less DPG in comparison with the WT strain could be due to decreased activity of the enzyme DPG synthetase, which forms DPG by condensation of two PG molecules (41). It is conceivable that our NIS^R strain has reduced DPG synthetase activity because the shifts in phospholipid composition are also observed when this strain is grown for various generations in the absence of nisin (data not shown).

It has been proposed that both electrostatic and hydrophobic interactions play an important role in the interaction between nisin and membranes (15, 33). Therefore, the interaction between nisin and pure lipids from the WT and the NIS^R strain was studied using the lipid-monolayer technique. This technique has proven to be a valuable tool in the study of membrane-active compounds (11). The monolayer results (Fig. 6) show that the pressure increase induced by nisin Z was higher for WT than for NIS^R lipid monolayers, reflecting qualitatively the difference in nisin sensitivity. At an initial pressure of 32 mN/m, which is thought to be a relevant surface pressure of biological membranes (12), the pressure increase is 6.0 and 9.5 mN/m for NIS^R and WT, respectively. The presence of the divalent cation Mg^{2+} resulted in a weaker interaction of nisin with monolayers of extracted lipids from the wild-type strain and its mutant. These ions also reduced nisin MIC values (data not shown). This is most likely a consequence of the interaction of Mg^{2+} with the negatively-charged head-groups of the phospholipids. This interaction might result in neutralization of the negatively-charged phospholipids (*i.e.* PG and DPG) in *L. monocytogenes*, which can reduce the efficiency of nisin action. Secondly, in the presence of divalent cations, a tighter packing of the acyl chains is introduced (21, 27), which can hinder nisin insertion. The effect of pH on the activity of nisin on lipid monolayers *i.e.* a lower pH reduces the nisin-lipid interactions, is in disagreement with the results obtained *in vivo* (1, 22, 23). An interpretation could be that a $\Delta\psi$ and/or ΔpH present in vegetative cells enhances nisin activity at acidic pH values.

In a previous study (13), it has been demonstrated that nisin penetrates more deeply into

lipid monolayers of DPG than those consisting of other lipids including PG, phosphatidylcholine (PC), phosphatidylethanolamine (PE), monogalactosyldiacylglycerol and digalactosyldiacylglycerol. Moreover, monolayers formed of lipid extracts of *Micrococcus flavus* were found to be much more strongly penetrated than were those of *L. monocytogenes* (13). *M. flavus* is extremely sensitive to nisin compared to several other Gram-positive bacteria (14) and, typically, the *M. flavus* membrane contains relatively high amounts of DPG and PG, *i.e.* 32% and 60% of the total phospholipid extracted respectively (13, 39). For the *L. monocytogenes* strain used by Demel *et al.* (13), these values were 7% DPG and 51% PG. Although both PG and DPG are negatively charged and therefore can interact with the cationic nisin molecule, the interaction with DPG is apparently much more strongly which may be linked to its high charge density and to its specific charge distribution (13, 19, 33). Using a nisin variant containing a tryptophan at position 30, Martin *et al.* (33) showed that the N-terminal part of nisin penetrated very deeply into the hydrophobic part of DPG-containing lipid bilayers.

In conclusion, the present study provides additional understanding of the mechanism of nisin resistance in *L. monocytogenes*. The results demonstrate that phospholipid headgroup alterations, particularly in the content of DPG, are the basis of a nisin-resistant variant of *L. monocytogenes* Scott A. However, the acquisition of nisin resistance in *Listeria* is probably a complex and multiple phenomenon and may differ among strains as appears from reports on alterations in fatty acid composition (35, 36) and cell wall changes (9, 32) in nisin-resistant listeriae.

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9

Variability in phenotypes of nisin-resistant *Listeria monocytogenes* strains

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Abstract

A number of phenotypic characters of five nisin-resistant *L. monocytogenes* strains which were acquired by increasing stepwise exposure to nisin, were compared with those of their parental strains. At pH 7.2, two nisin-resistant variants grew slower than their nisin-sensitive counterparts both at 7°C and 30°C, whereas the other three nisin-resistant strains exhibited similar growth rates as their parent strains at both temperatures. However, one of the three last mentioned mutants grew much slower at pH 5.6 compared to the original strain. Three of the five nisin-resistant variants demonstrated a reduced sensitivity towards the peptidoglycan hydrolytic enzyme mutanolysin compared with their nisin-sensitive wild-type strains, whereas one of the nisin-resistant strains showed an increased sensitivity to mutanolysin in comparison with its parent strain. The *L. monocytogenes* strains manifested completely unpredictable susceptibility towards pediocin. The nisin-sensitive strains were either sensitive or resistant to pediocin and the same was valid for the nisin-resistant strains without indications for either cross-sensitivity or cross-resistance to nisin and pediocin. The results show that the mechanism by which *L. monocytogenes* gains nisin resistance may vary among strains and suggest that nisin resistance in *L. monocytogenes* is a complex multifaceted phenomenon. Importantly, all resistant strains became increasingly sensitive to nisin at pH 5.6, a representative pH for many foods, as compared to pH 7.2. With skimmed milk as a model it was demonstrated that nisin and pediocin are more effective in a mixture than individually against nisin-sensitive and nisin-resistant *L. monocytogenes* Scott A. The use of nisin in a cocktail with other bacteriocins or in combination with other mild preservation techniques like low pH might therefore be an efficient preservation strategy.

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Introduction

The presence of *L. monocytogenes* in foods that do not receive a severe processing treatment is unavoidable since the pathogen is ubiquitous in the environment. In addition, these minimally processed foods generally rely on refrigeration as the main preservation method allowing the psychrotrophic pathogen to multiply. The addition of bacteriocins that inhibit the growth of *L. monocytogenes* (and other food-borne pathogens) is therefore considered to increase the microbial safety of these foods. Although numerous bacteriocin-producing lactic acid bacteria inhibitory to *L. monocytogenes* have been isolated from food, nisin is the only candidate since none of the other bacteriocins known to date has a fully approved legal status as a food additive (20). Widespread and constant application of nisin may however select for nisin-resistant pathogens that would hamper the efficacy of nisin.

Acquisition of nisin resistance has been reported to occur in different strains of *L. monocytogenes* yielding cultures which are 2 to 10 times more resistant to nisin than the original cultures (6, 8, 12, 17, 18, 27). Some bacterial species have been shown to be resistant to nisin due to their ability to produce an enzyme which inactivates nisin (14, 22). To date such an enzyme has not been detected in nisin-resistant *L. monocytogenes* strains (6, 18). All resistance mechanisms described for *L. monocytogenes* involve alterations in the cell envelope, either at the level of the cytoplasmic membrane (17, 18, 19, 27) or at the cell wall (6, 7, 15). These alterations are thought to affect the presence or accessability of attachment sites hindering the incorporation of nisin into the membrane to produce pores, thus preventing rapid efflux of small cytoplasmic components and subsequent disruption of the proton motive force (PMF).

Our previous study focussed on the characterization of the mechanism of nisin resistance in a variant of *L. monocytogenes* Scott A which was trained to resist relatively high nisin concentrations. The nisin resistance in this particular strain was shown to be attributable to a reduction in the diphosphatidylglycerol (DPG) content of the cytoplasmic membrane (27). The present investigation was performed to determine some properties of a range of nisin-resistant *L. monocytogenes* strains and their parental strains, including the nisin-sensitive Scott A and its nisin-resistant derivative, in order to provide a better base of knowledge for the application of nisin in foods. Having compared the sensitivity of the strains towards nisin at different pH values, their growth characteristics at refrigeration temperature (7°C) and optimal growth temperature (30°C), the action of the cell wall lytic enzyme mutanolysin on nisin-sensitive and nisin-resistant cells, and their possible cross-resistance to the bacteriocin pediocin, it appears that there exists a great variability in the phenotypes of nisin-resistant *L. monocytogenes* strains. The effect of a combination of nisin and pediocin on nisin-sensitive and nisin-resistant *L. monocytogenes* Scott A was studied in skimmed milk to investigate possible synergistic inhibitory effects of these bacteriocins.

Materials and Methods

***L. monocytogenes* strains.** *L. monocytogenes* 4B, 669, and 13 are isolates from food or food environments and were obtained from the culture collection of NESTEC (Switzerland). *L.*

monocytogenes Scott A was from our own culture collection. Nisin-resistant derivatives of the four strains were acquired by increasing stepwise exposure to nisin (0.6, 1.0, 1.6, 2.6, 3.4, 5.0, 7.0, 10, 15, 30 mg/l) in brain heart infusion (BHI) broth, which has a pH of 7.2. In addition, a nisin-resistant mutant of the Scott A strain was selected in BHI broth, which was adjusted to pH 5.6 with HCl, by increasing the nisin concentration from 0.05 mg/l to 3 mg/l in 20 steps. Strains were stored at -80°C in BHI broth containing 20% (vol/vol) glycerol and, although the nisin resistance phenotypes were stable when transferred in nisin-free broth for several generations, the nisin-resistant variants were maintained in the presence of nisin. Nine different *L. monocytogenes* strains were used throughout this study: *L. monocytogenes* Scott A (WT Scott A), *L. monocytogenes* 4B (WT 4B), *L. monocytogenes* 669 (WT 669), *L. monocytogenes* 13 (WT 13), and their nisin-resistant variants isolated at pH 7.2, which are abbreviated as NIS^R Scott A, NIS^R 4B, NIS^R 669 and NIS^R 13, respectively. The ninth strain is the nisin-resistant variant of *L. monocytogenes* Scott A isolated at pH 5.6 ($NIS^R_{pH5.6}$ Scott A).

Bacteriocin preparations. Stock solutions of nisin (1 g/l) were prepared by dissolving nisaplin (Sigma, St. Louis, Mo., USA), which contains 2.5% nisin (wt/wt) in sterile 0.02 M HCl and were diluted to the appropriate concentrations just before use. Pediocin PA-1 was prepared from the cell-free supernatant of the producer strain *Pediococcus acidilactici* PAC 1.0 (5) after overnight growth in MRS broth at 30°C. The supernatant containing pediocin was concentrated 10-fold by freeze drying and resuspended in water. After adjustment to pH 6.5 and sterilization through a 0.2-μm-pore-size filter (Schleicher & Schuell GmbH, Dassell, Germany), this preparation was used directly in further experiments.

Determination of nisin minimal inhibitory concentration (MIC) values. *L. monocytogenes* strains were grown overnight at 30°C in BHI broth, inoculated with approximately 10^5 cells per ml into the different wells of a microtitre plate which contained fresh BHI with increasing nisin concentrations. BHI was adjusted to the appropriate pH with HCl where indicated. MICs were determined as being the lowest nisin concentration which prevented growth after incubation for 24 hours at 30°C.

Growth measurements. Growth experiments were performed in BHI broth at 7°C and 30°C using an inoculum level of about 10^4 to 10^5 *L. monocytogenes* cells per ml. Changes in absorption were measured at 620 nm (OD_{620}) in a microtitre plate reader (Type 400FW or 340 ATTC, SLT-Instruments, Salzburg, Austria).

Measurement of acid tolerance response (ATR). To establish whether the nisin-resistant mutant of the Scott A strain selected at pH 5.6 has increased natural acid resistance the following experiment was conducted. The method essentially described by O'Driscoll *et al.* (21) was used with slight modifications. Overnight cultures of *L. monocytogenes* Scott A strains grown in BHI broth were inoculated into fresh broth (2% inoculum) and allowed to grow to an OD_{620} of approximately 0.2. Triplicate samples of each culture were centrifuged and resuspended in BHI (pH 7.2) and in BHI adjusted to pH 2 and pH 5 using HCl, and incubated for 1 h at 30°C. Subsequently, cells were centrifuged and resuspended for 1 h in BHI acidified to pH 2 with HCl and viable counts were determined by plating serial dilutions onto tryptic soya agar (TSA) plates. Plates were incubated for 48 h at 30°C, after which the colonies were counted.

Mutanolysin action. Cells of *L. monocytogenes* grown in BHI broth at 30°C were harvested at an OD_{620} of 0.6, washed twice in 50 mM potassium 2-(*N*-morpholino)ethanesulfonic acid (K-MES) buffer (pH 6.5) and resuspended to a final OD_{620} of approximately 1. Following incubation for 30 min at 37°C, 5 U/ml of mutanolysin (from *Streptomyces globisporus* ATCC 21553, Sigma M-9901) was

added and the turbidity was recorded at 620 nm using a Novaspec II spectrometer (Pharmacia Biotech) every 30 min for 5h.

Determination of pediocin sensitivity. The activity of the pediocin preparation towards *L. monocytogenes* was determined by agar well-diffusion assays. Specifically, 150 μl from an overnight culture of *L. monocytogenes* in BHI was mixed with 15 ml of BHI agar held at 45°C. The agar was dispensed into round sterile 8.5 cm diameter petri dishes and after solidification, wells were made by removing the agar using a 6 mm diameter glass tube. Subsequently, 30 μl of the pediocin preparation was dispensed in individual wells. Inhibition zones were measured after incubation at 30°C for 24 h.

Effect of nisin and pediocin on membrane potential. Cells of *L. monocytogenes* strains were grown in BHI broth at 30°C and harvested during logarithmic growth at an OD_{620} of 0.7, washed twice in 50 mM potassium *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (K-HEPES) buffer (pH 7.2). The $\Delta\psi$ (inside negative) was monitored with the potential-sensitive fluorescent probe 3,3-dipropylthiadicarbocyanine ($DiSC_3[5]$) (excitation wavelength, 643 nm; emission wavelength, 666 nm). Cells were diluted to a final OD_{620} of 0.25 in 50 mM (K-HEPES) buffer (pH 7.2), glucose was added to a final concentration of 0.2 % (wt/vol), and the effects of nisin and pediocin on the membrane potential were monitored in the presence of nigericin (1 μM) to prevent the generation of a transmembrane pH gradient. Fluorescence measurements were performed using a Perkin-Elmer LS 50 spectrofluorimeter at 30°C with continuous stirring.

Nisin and pediocin action on ATP levels. Cells were prepared as described above, except that they were washed in 50 mM potassium phosphate (pH 7.2), and resuspendend in this buffer to a final OD_{620} of about 20. Cells were diluted 20-fold in the buffer in a temperature controlled stirred vessel, glucose was added to a final concentration of 0.5% (wt/vol), and after 10 min of incubation, either nisin or pediocin was incorporated in the assay mixture. Aliquots of the incubation mixture were measured for total and external ATP levels at appropriate time intervals, internal levels were estimated by substracting the external from the total ATP levels. Cells were lysed using dimethylsulfoxide and ATP concentrations were determined using the Lumac (Landgraaf, the Netherlands) luciferase bioluminescence assay, the amount of omitted light was recorded with a Lumac biocounter M2500.

Growth of nisin-sensitive and nisin-resistant *L. monocytogenes* Scott A in skimmed milk in the presence of nisin and/or pediocin. Commercially available sterile skim milk was divided into 10 ml fractions in 100-ml Erlenmeyer flasks and nisin and/or pediocin was added at the concentrations indicated. *L. monocytogenes* Scott A and its nisin-resistant mutant were then inoculated to give an initial concentration of approximately 10^5 cells per ml. Flasks were incubated at 30°C in a shaker-incubator and samples were taken after various time intervals. Colony forming units (CFUs) were determined by plating appropriate dilutions on TSA and subsequent counting after incubation for 48 h at 30°C.

Chemicals. BHI, MRS and TSA were from Difco Laboratories (Detroit, MI, USA). The fluorescent dye $DiSC_3[5]$ was obtained from Molecular Probes Europe B.V. (Leiden, The Netherlands). All other chemicals were reagent grade and obtained from commercial sources.

Results

Nisin MIC values in BHI broth at different pH values. Nisin MIC values for WT Scott A and NIS^R Scott A were determined in BHI broth in the pH range 5.0 to 8.0 (Fig. 1). At

pH 7.2, which is the normal pH of BHI broth, the WT Scott A strain had a MIC of 3.2 ± 0.2 mg/l compared with 37.5 ± 2.5 mg/l for NIS^R Scott A. The nisin MIC values decreased gradually with pH for both strains. At lower pH values, the MICs for WT and NIS^R converged and at an external pH of 5.0, the MIC values were equal, *i.e.* 0.1 mg/l. It was established that WT and NIS^R showed no differences in growth behaviour in BHI broth without nisin at a particular pH (data not shown).

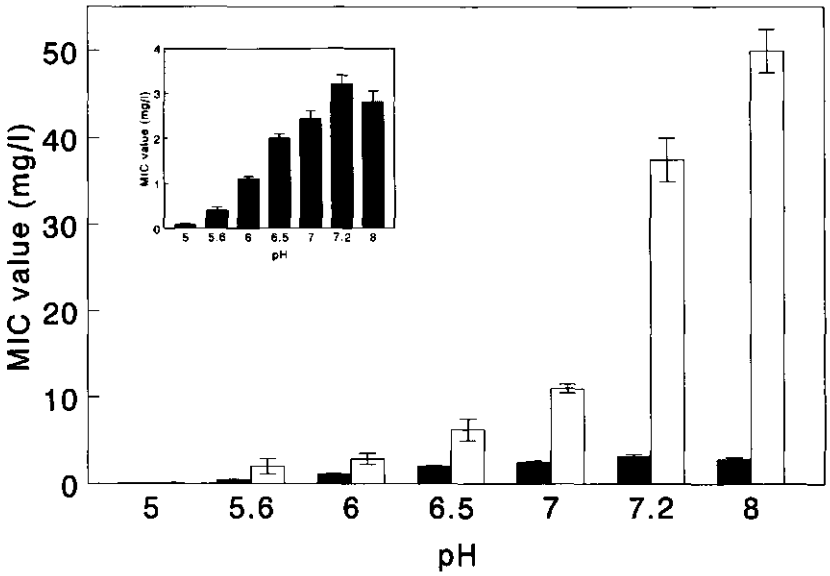

Figure 1. Minimal inhibitory concentration (MIC) values of nisin for wild-type (*black bars*) and nisin-resistant (*white bars*) *L. monocytogenes* Scott A in BHI broth at different pH values. MIC values were determined as described in Materials and Methods and are the means ± standard deviation of triple determinations. *Inset:* MIC values for WT Scott A on a larger Y-scale.

MIC values of the other strains were determined at pH 7.2 and pH 5.6, the latter pH is considered to reflect the situation in many foods. NIS^R 4B, NIS^R 669, NIS^R 13 had an increased resistance to nisin compared with their parent strains (Fig. 2). At pH 7.2, the nisin MIC value for NIS^R 4B is, like that of NIS^R Scott A, more than 10 times the original MIC value, whereas NIS^R 669 and NIS^R 13 were only two to three times as resistant to nisin as their parental strains. Nisin MIC values of the strains were significantly reduced at pH 5.6 (Fig. 2). The resistant variants of *L. monocytogenes* 669 and 13 grew much slower at pH 5.6 compared to their sensitive counterparts, resulting in a higher MIC value for the WT than for the NIS^R strains. MIC values found for NIS$^R_{pH5.6}$ Scott A at pH 5.6 and 7.2 were 2.7 ± 0.2 and 38.8 ± 1.8 respectively (data not shown), and thus in the same order of magnitude as those recorded for NIS^R Scott A, indicating that the resistance to nisin at a particular pH is independent of the pH at which the strain was trained to nisin.

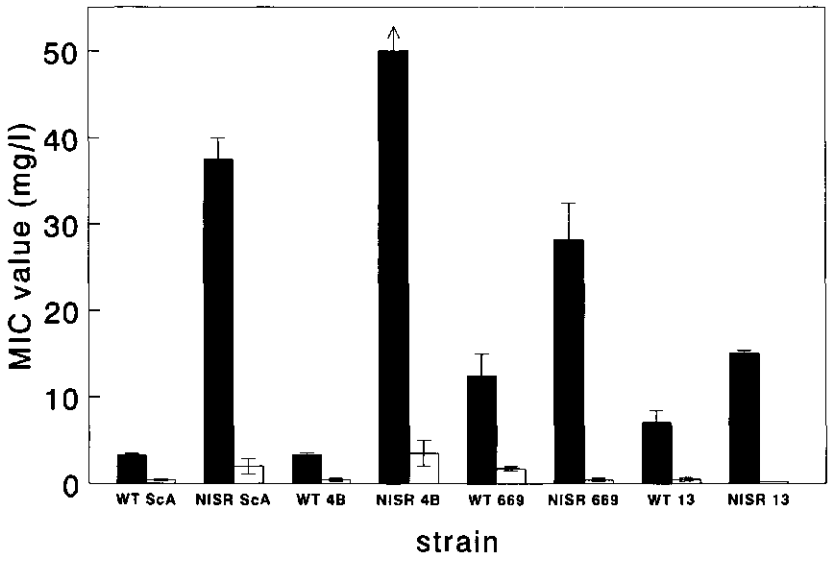

Figure 2. Nisin MIC values of WT and NIS^R *L. monocytogenes* strains at pH 7.2 (*black bars*) and pH 5.6 (*white bars*) in BHI broth. Values are the means ± standard deviation of triple determinations.

ATR in *L. monocytogenes* Scott A strains. Early-log-phase cultures of all three *L. monocytogenes* Scott A strains (WT, NIS^R and NIS$^R_{pH5.6}$) grown in BHI at pH 7.2 were very sensitive to a sudden change to pH 2, a 5-log-unit reduction in cell number was observed after 1 h for all three strains. Shifting the cultures for 1 h to pH 5 prior to exposure to pH 2 for 1 h, resulted in about 1-log-unit reduction for all three strains (data not shown). These results indicate that tolerance to low pH can be induced by exposure to sublethal pH for *L. monocytogenes* Scott A strains, but give no indications for increased natural (*i.e.*, constitutively expressed) acid tolerance in NIS$^R_{pH5.6}$ Scott A as compared to the other two strains.

Growth of *L. monocytogenes* strains at 7°C and 30°C. Growth rates of the WT and NIS^R strains at 30°C were mutually the same except for strain 669, the growth rate of NIS^R 669 was slower than that of WT 669 and in addition a much longer lag time was observed for NIS^R 669 (Fig 3). At 7°C, WT Scott A and NIS^R Scott A again showed virtually the same growth characteristics and this was also true for WT 4B and NIS^R 4B. Both WT 13 and NIS^R 13 refused to grow at 7°C, whereas the growth behaviour of NIS^R 669 was again different from that of WT 669, a longer lag time and a slower growth rate were encountered for the nisin-resistant variant (Fig 4). The NIS$^R_{pH5.6}$ Scott A strain exhibited a reduced growth rate compared to WT Scott A and NIS^R Scott A, both at 30°C and 7°C.

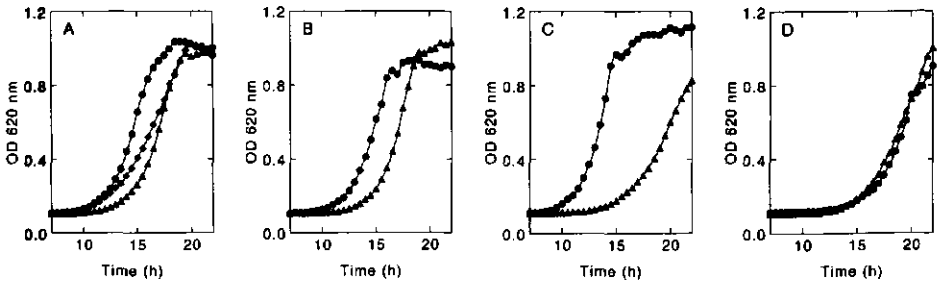

Figure 3. Growth of *L. monocytogenes* strains at 30°C in BHI broth. (A), *L. monocytogenes* Scott A; (B), *L. monocytogenes* 4B; (C), *L. monocytogenes* 669, and (D) *L. monocytogenes* 13. The growth of WT and NIS^R variants are represented in all graphs by (●) and (▲), respectively. Growth of $NIS^R_{pH5.6}$ Scott A is shown in panel A (◆). Growth was determined at an OD_{620} in microtiter plates, the OD values were normalized to a path length of 1 cm.

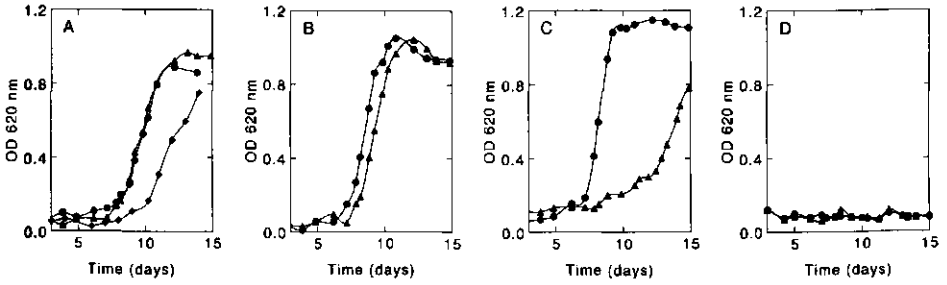

Figure 4. Growth of *L. monocytogenes* strains at 7°C in BHI broth. Symbols are the same as in figure 3.

Action of mutanolysin on nisin-sensitive and nisin-resistant *L. monocytogenes*. In an earlier study (27), it was established that the WT Scott A and NIS^R Scott A showed a comparable sensitivity to lysozyme. The effect of mutanolysin, which is like lysozyme a peptidoglycan hydrolytic enzyme, on the nine *L. monocytogenes* strains is shown in Fig. 5. As anticipated, cells of WT Scott A and NIS^R Scott A were lysed at almost an equal rate upon addition of mutanolysin (Fig. 5A). However, $NIS^R_{pH5.6}$ Scott A showed an increased sensitivity towards mutanolysin compared with the two other Scott A strains. The nisin-resistant variants of 669, 4B and 13 were lysed at a slower rate than their nisin-sensitive parent strains. Particularly NIS^R 669 was highly resistant to mutanolysin as compared to WT 669 (Fig. 5C).

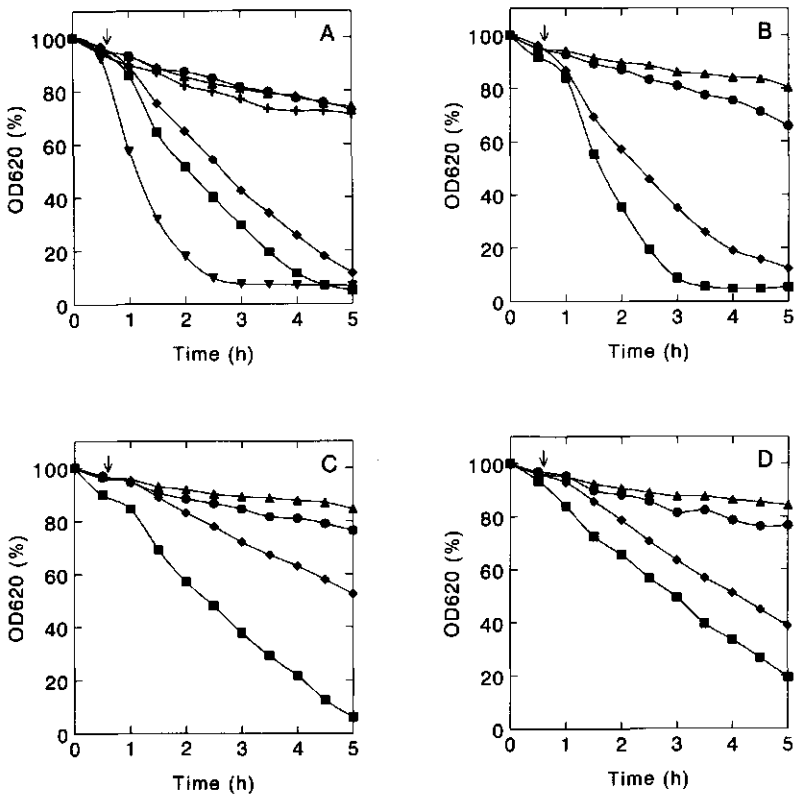

Figure 5. Action of mutanolysin on washed cell suspensions (OD_{620}) of *L. monocytogenes* strains: Scott A (A), 4B (B), 669 (C), and 13 (D). The decrease in OD_{620} was recorded in K-MES (pH 6.5) in the absence (control) or presence of mutanolysin (5U/ml) for WT and NIS^R strains. Symbols: (●), WT, control; (■) WT, mutanolysin; (▲), NIS^R, control; (◆), NIS^R, mutanolysin. The effect of mutanolysin on $NIS^R_{pH5.6}$ Scott A is included in panel A, (+), control; (▾), mutanolysin.

Sensitivity of *L. monocytogenes* to pediocin. WT Scott A and NIS^R Scott A were examined for sensitivity to nisin and pediocin in a agar well-diffusion test (Fig. 6). Surprisingly, WT Scott A appeared to be insensitive to pediocin whereas this bacteriocin gave a significant zone of inhibition against NIS^R Scott A. This result prompted us to determine the activity of pediocin towards the other nisin-sensitive and nisin-resistant *L. monocytogenes* strains (Table 1). For 669 just the reverse was found as for Scott A, WT 669 appeared to be sensitive to pediocin in contrast with NIS^R 669. NIS^R 4B is sensitive to pediocin as well as the parental strain and both WT 13 and NIS^R 13 are insensitive at the pediocin concentration applied. In addition, no zone of inhibition was visible against $NISR^R_{pH5.6}$ Scott A.

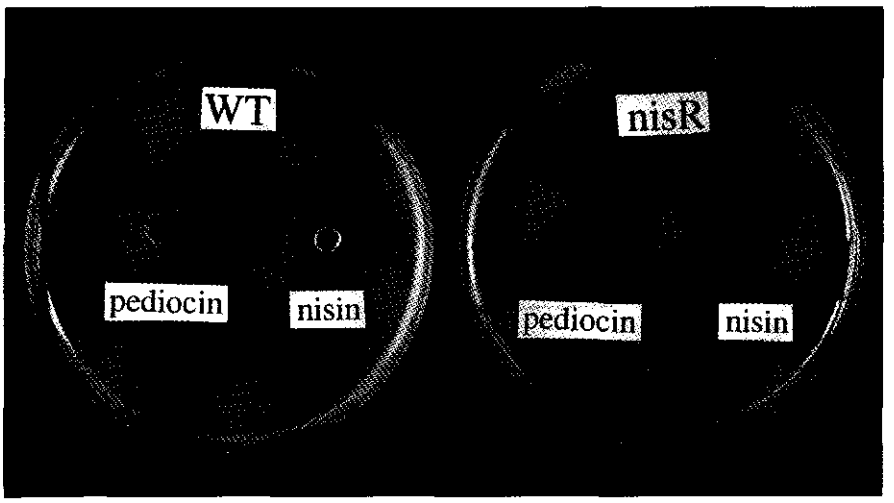

Figure 6. Inhibition zones produced by nisin and pediocin against *L. monocytogenes* WT Scott A (*left*) and NIS^R Scott A (*right*) in a agar well-difffusion test. Wells contained 30 μl of bacteriocin solutions of pediocin (10 times concentrated cell-free supernatant of the producer strain *Pediococcus acidilactici* PAC 1.0 adjusted to pH 6.5) or nisin (10 mg/l) as indicated. Plates were incubated at 30°C for 24 h.

Table 1. Activity of pediocin towards *L. monocytogenes*[a]

L. monocytogenes strain	WT	NIS^R	NIS$^R_{pH5.6}$
Scott A	0	18	0
669	18	0	
4B	20	18	
13	0	0	

[a] Results are given in diameters (mm) of inhibition with 30 μl of pediocin extract (10 times concentrated cell-free supernatant of the producer strain *Pediococcus acidilactici* PAC 1.0) adjusted to pH 6.5 in a BHI well-diffusion assay. Plates were incubated at 30°C for 24 h.

Influence of nisin and pediocin on membrane potential in *L. monocytogenes* cells. The $\Delta\psi$ of the WT strains was more severely affected upon addition of 0.33 mg/l nisin compared with their NIS^R variants (Fig. 7). The addition of pediocin (10 μl of the concentrated cell-free supernatant of the producer strain in a total volume of 3 ml) to NIS^R Scott A severely

reduced the $\Delta\psi$, whereas the $\Delta\psi$ in WT Scott A remained unaffected upon pediocin addition (Fig. 7A). For *L. monocytogenes* 669 just the reverse was found, the $\Delta\psi$ in NIS^R 669 was not influenced in contrast with that in WT 669 (Fig. 7C). Both WT 4B and NIS^R 4B showed dissipation of the $\Delta\psi$ upon pediocin addition, the effect was more pronounced for the former strain. The membrane potentials in WT 13 and NIS^R 13 were not affected by pediocin supplementation. Noteworthy, the effect of pediocin on the membrane potential corresponds with the activity of pediocin towards *L. monocytogenes* as determined by the agar well-diffusion assay.

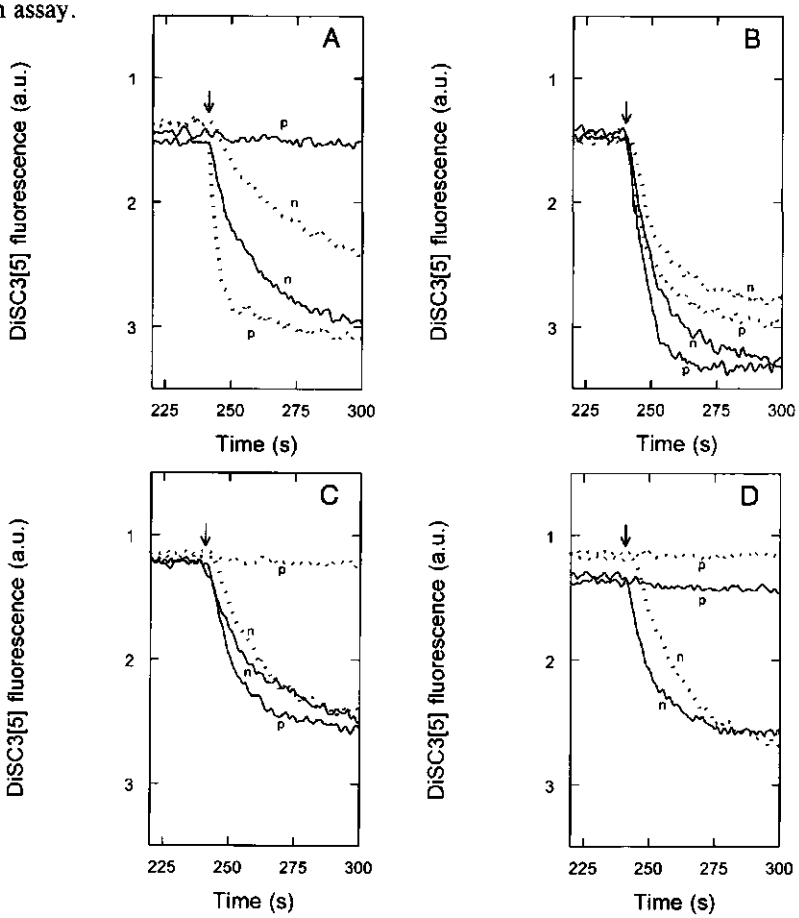

Figure 7. Effect of nisin and pediocin on the membrane potential of *L. monocytogenes* Scott A (A), 4B (B), 669 (C), and 13 (D) in K-HEPES buffer (pH 7.2). Assays were performed with WT (—) and NIS^R (‚‚‚‚) variants of the strains using a final OD_{620} of 0.25. $DiSC_3[5]$ (5 μM) and 0.5% (wt/vol) glucose were added to the cells, followed by the addition of 1 μM nigericin. At the times indicated by the arrows, either nisin (**n**, 0.33 mg/l) or pediocin (**p**, 10 μl of the 10 times concentrated cell-free supernatant of the producer strain *Pediococcus acidilactici* PAC 1.0 adjusted to pH 7.2 in a total volume of 3 ml) was added. Valinomycin (1 μM) was used as a control (complete dissipation of $\Delta\psi$), resulting in approximately 4 arbitrairy fluorescence units for the different strains (not shown).

Effect of nisin and pediocin on ATP levels of *L. monocytogenes* Scott A and 669 . The addition of 2.5 mg/l nisin to energized cells of WT Scott A resulted in an immediate decrease in intracellular ATP. Only about 40% of the total ATP was found in the external medium which indicates that nisin addition not only resulted in ATP efflux but additionally in hydrolysis of ATP. The ATP pool of NIS^R Scott A was only slightly reduced upon adding 2.5 mg/l nisin and this strain was able to restore ATP production within about 5 min (Fig. 8A). A concentration of 2.5 mg/l nisin caused a complete depletion of ATP in both WT 669 and NIS^R 669 (Fig. 8C), whereas adding nisin at a concentration of 1 mg/l totally depleted cytoplasmic ATP in WT 669, whereas NIS^R 669 was able to revive after 20 min (not shown).

The addition of pediocin to energized cells of NIS^R Scott A resulted in a gradual decrease of intracellular ATP, whereas little ATP efflux was recorded (Fig 8B). Pediocin induced ATP efflux from WT 669 whereas ATP hydrolysis not occurred since the amount of total ATP just before the addition of the bacteriocin equaled the amount of external ATP 15 min after pediocin addition. The finding that ATP pools in NIS^R Scott A and WT 669 are affected by pediocin in contrast with WT Scott A and NIS^R 669 (Fig. 8B and 8D) is in line with the activity of pediocin towards *L. monocytogenes* as determined by the agar well-diffusion assay.

Growth of WT Scott A and NIS^R Scott A in skimmed milk. The effect of nisin and pediocin on growth of WT Scott A and NIS^R Scott A in foods was studied using skimmed milk as a model (Fig. 9). The addition of nisin at a concentration of 10 mg/l to skimmed milk that had been inocculated with WT Scott A resulted in a decrease in colony forming units below the detection limit. Adding half of the amount of nisin (*i.e.* 5 mg/l) resulted in an initial decrease of 2 log units, whereafter growth restoration was observed. The addition of 5 % (vol/vol) of the pediocin extract had no effect on the growth of WT Scott A in skimmed milk, whereas the presence of 5% (vol/vol) of the pediocin extract plus 5 mg/l nisin resulted in a complete kill of WT Scott A (Fig. 9A). Adding nisin (10 mg/l) to skimmed milk containing NIS^R Scott A resulted in a reduction of CFUs over about half a log unit after which growth resumed. Pediocin (10% (vol/vol)) had a more dramatic effect on NIS^R Scott A, CFUs were reduced from 1.8×10^4 CFU/ml to about 30 CFU/ml. When using half of the concentrations of both bacteriocins in a mixture (*i.e.* 5 mg/l nisin and 5% (vol/vol) pediocin), NIS^R Scott A was killed completely (Fig. 9B). The effect of both bacteriocins was also tested in skimmed milk inoculated with both WT Scott A and NIS^R Scott A and the total number of *L. monocytogenes* Scott A was monitored (Fig. 9C). Just after addition of 10% (vol/vol) pediocin a slight decrease in CFUs was observed followed by resumption of growth. Addition of 10 mg/l nisin resulted in an initial decrease in viable cells (about 2 log units), after which growth resumed. Most likely, the NIS^R cells survived the nisin treatment in contrast with the WT cells. None of the cells remained alive upon the addition of a combination of nisin and pediocin containing the same concentrations as in the individual experiments. Adding a combination of these bacteriocins using half of the concentrations resulted in a decrease from 2×10^4 CFU/ml to 30 CFU/ml, and after about 9 h, an increase in cell numbers was observed.

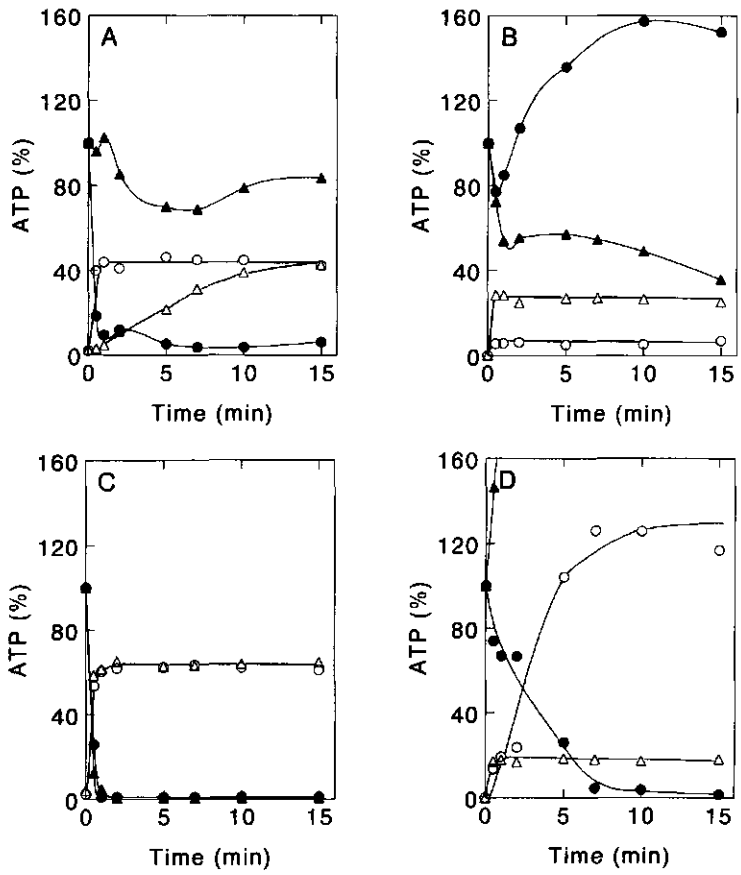

Figure 8. Effect of nisin (A, C) and pediocin (B, D) on cytoplasmic (●, ▲) and external ATP levels (○, Δ) in *L. monocytogenes* Scott A (A, B) and *L. monocytogenes* 669 (C, D). Following preincubation of cells (OD_{620}, 1) in 50 mM potassium phosphate (pH 7.2) containing 0.5% (wt/vol) glucose for 10 minutes at 30°C, nisin (2.5 mg/ml) or pediocin (1 ml of the 10 times concentrated cell-free supernatant of the producer strain *Pediococcus acidilactici* PAC 1.0 adjusted to pH 7.2 in a total volume of 2 ml). The total absolute amount of ATP just before the addition of the bacteriocins was put at 100%. *Circles* and *triangles* symbols represent ATP concentrations in WT and NIS^R strains respectively.

Discussion

This study demonstrates that *L. monocytogenes* strains which gained nisin resistance through serial passages in nisin-containing medium, may exhibit phenotypic characters that differ from that of the parental strain. The susceptibility towards nisin, pediocin and mutanolysin differed greatly between the strains and differences in growth characteristics were observed both between the WT strains on the one hand and between the WT and NIS^R

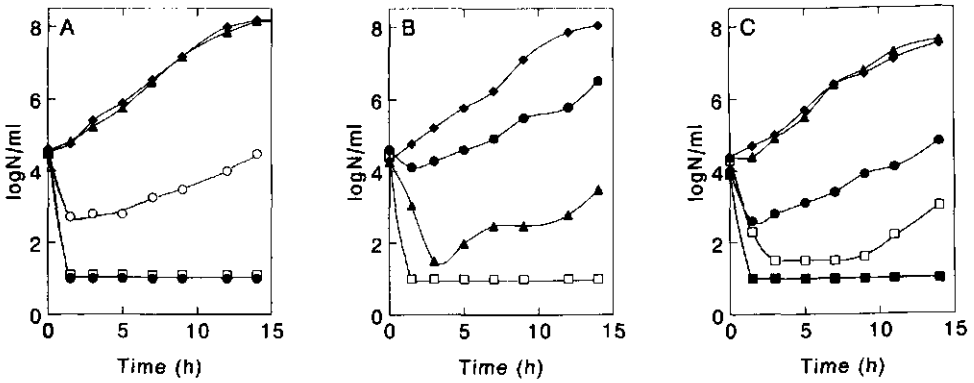

Figure 9. Growth of *L. monocytogenes* Scott A in sterile skimmed milk. (A), WT Scott A; (B), NIS^R Scott A; (C) WT Scott A and NIS^R Scott A were inoculated in the absence (◆) or presence of 10 mg/l nisin (●), 10 % (vol/vol) pediocin (▲), 10 mg/l nisin plus 10 % (vol/vol) pediocin (■), 5 mg/l nisin plus 5 % (vol/vol) pediocin (□) or 5 mg/l nisin (○), and colony forming units (CFUs) were recorded in time. A logN/ml value of 1 corresponds with the detection limit.

variants of one strain on the other hand. This suggests that the mechanism by which *L. monocytogenes* acquires nisin resistance may vary between strains. The finding that a nisin-resistant variant of *L. monocytogenes* Scott A which was trained to nisin at pH 7.2 had different properties than a nisin-resistant variant of the same strain which was trained to nisin at pH 5.6 suggests that nisin resistance mechanisms might also be dependent on the conditions under which the strain obtained resistance.

The growth rate of the nisin-resistant variant of *L. monocytogenes* 669 was significantly decreased as compared with the parental strain both at 30 and 7°C, whereas NIS^R Scott A, NIS^R 4B and NIS^R 13 showed similar growth behaviour as their WT counterparts at both temperatures. $NIS^R_{pH5.6}$ Scott A also exhibited a slower growth rate than the original strain. Slower growth of nisin-resistant variants of *L. monocytogenes* in comparison with their parent strains has been described before (17, 18). The nisin resistance mechanism in these strains involves changes in fatty acid composition that result in a more rigid membrane which apparently does not permit normal growth. Recently, it has also been demonstrated that nisin resistance in some *Listeria* strains is based on cell wall changes (7, 15). NIS^R 669 showed increased resistance to the peptidoglycan hydrolytic enzyme mutanolysin as compared to WT 669, while $NIS^R_{pH5.6}$ Scott A showed increased sensitivity to this enzyme as compared to WT Scott A. The latter results suggests that these two resistant variants have altered their cell wall, whereas the slower growth of these strains might additionally indicate changes in the fatty acid composition of the cytoplasmic membrane. Considering the difference in sensitivity towards mutanolysin between nisin-sensitive and nisin-resistant cells of *L. monocytogenes* 4B and 13, it is plausible that cell wall changes also play a role in the mechanism of nisin resistance in these two strains. Moreover, the membrane potential in the nisin-resistant variant of strain 13 was increased compared to the original strain (Fig. 7), indicating that

bioenergetics is affected in this strain and, additionally, this strain grew very slow at pH 5.6 as compared with its sensitive counterpart. These data suggest that several aspects may contribute to the resistance mechanism in this strain. The above considerations in combination with the results of our previous work in which we demonstrated that nisin resistance in NIS^R Scott A is mainly attributable to a reduction in DPG content in the cytoplasmic membrane (27), make clear that the nisin resistance in *L. monocytogenes* is a complex multifaceted phenomenon.

The exposure of the nine strains to pediocin resulted in rather striking results. The agar well-diffusion assays as well as the $\Delta\psi$ and ATP measurements clearly indicated that NIS^R Scott A, WT 669, WT 4B and NIS^R 4B were highly susceptible for pediocin whereas the other strains were insensitive. These results indicate that the strains show no consistent cross-sensitivity or cross-resistance to the bacteriocins nisin and pediocin, which seems important with regard to the application of bacteriocin cocktails. Rekhif *et al.* (23) have demonstrated that mutants of *L. monocytogenes* that acquired resistance to the inhibitory action of either mesenterocin 52, curvaticin 13 or plantaricin C19, which all belong to the pediocin-like class of bacteriocins (13), showed cross-resistance to the three bacteriocins, but not to nisin. However, Mazzota *et al.* (16) recently demonstrated that nisin-resistant isolates of *Clostridium botulinum* also had increased resistance to pediocin PA-1, bavaricin MN, plantaricin BM, and leconocin S, which suggests that application of more than one bacteriocin would not circumvent bacteriocin resistance for this pathogen. When testing the effectiveness of nisin and pediocin in combination to inhibit the growth of *L. monocytogenes* WT Scott A and/or NIS^R Scott A in skimmed milk, it became clear that in a cocktail, bacteriocins may have a synergistic effect. Possibly, cells that resist one of the bacteriocins are still somewhat injured making them more susceptible to the other bacteriocin. Taken together, the use of nisin and pediocin in a cocktail not only provides greater inhibitory levels, but may also be more efficient against the development of resistant variants of *L. monocytogenes*.

The results regarding the susceptibility of the strains towards pediocin give, in addition, indications for different nisin resistance mechanisms in the *L. monocytogenes* strains since nisin and pediocin have different mechanisms of action. Although both bacteriocins form pores in the membrane of target cells causing a rapid efflux of small cytoplasmic molecules and ions from the target cells and a collapse of the PMF, nisin is believed to inhibit sensitive organisms in a voltage-dependent way in absence of a protein receptor, whereas pediocin has been suggested to act in a voltage-independent protein receptor-mediated way (1, 2, 5, 9, 10, 24). However, Chen *et al.* (4) recently suggested that electrostatic interactions play a dominant role in the binding of pediocin to membranes since it was demonstrated that pediocin PA-1 can permeabilize lipid vesicles in the absence of a protein receptor. In our previous work we showed that nisin-resistance in NIS^R Scott A is based on a reduction in the diphosphatidylglycorol (DPG) content of the cytoplasmic membrane (27). It seems unlikely that this alteration would result in sensitivity to pediocin, since this bacteriocin is, like nisin, cationic in nature (4,13). It is therefore assumed that other factors, such as exposure of possible receptor sites, play a role in enhanced sensitivity of the NIS^R Scott A to pediocin.

It has been proposed that nisin forms pores in the cytoplasmic membrane with diameters

ranging from 0.2 to 1.0 nm in black lipid membranes (25) which would allow solutes with molecular masses up to 0.6 kDa to pass (molecular weight ATP is 0.5 kDa). The size of the pores formed during the action of pediocin is concentration dependent, relatively low concentrations induce efflux of small ions like protons, potassium and phosphate, whereas at higher concentrations larger pores are formed allowing the passage of compounds with molecular masses up to 9.6 kDa (3, 5). The above rationalizes why nisin caused growth inhibition, depletion of the intracellular ATP pools and collapse of the $\Delta\psi$ in sensitive cells at concentrations that are in the same order of magnitude (note that the OD value in the ATP experiments was 4 times higher than in the $\Delta\psi$ assays), whereas pediocin induced dissipation of the $\Delta\psi$ at a concentration that was about 50 times lower than that needed to induce ATP leakage in sensitive cells. Moreover, it might explain the different effect of pediocin on ATP pools in the pediocin-sensitive strains NIS^R Scott A and WT 669. Probably, the pores formed by pediocin in WT 669 are large allowing ATP leakage whereas in NIS^R Scott A, the pores allow efflux of only small molecules. In the latter case, ATP hydrolysis might result from the attempt to maintain a PMF or could be due to phosphate efflux (1, 3, 11).

At physiological pH, the nisin MIC value for NIS^R Scott A was about 10 times higher than that of WT Scott A. Lowering the external pH, gradually brought about a similar sensitivity to nisin for WT and NIS^R Scott A (Fig. 1). Low external pH values result in changes in the net charge of nisin due to protonation of histidine residues, which may enhance its activity. It is unlikely that changes in the membrane itself contribute to the decreased MICs at lower pH values, because the pK values of the acidic lipid molecules lie outside the pH range examined (13, 28). The finding that at low pH the resistant variants become increasingly sensitive to nisin as compared to the wild type strains, is an important observation with regard to the situation in foods, since most foods have a slightly acidic pH (about pH 5.6). However, we demonstrated that it was still possible to generate a nisin-resistant isolate of *L. monocytogenes* Scott A at pH 5.6, which was as resistant to nisin at pH 5.6 and at pH 7.2 as the mutant that was obtained at pH 7.2. De Martinis *et al.* (8) demonstrated that a combination of food-preservation hurdles (low pH, low temperature, high salt) diminishes the likelihood of nisin-resistant variants to emerge, whereas those factors on their own did not reduce the frequency at which resistance arises.

Recently, it has been suggested that low pH conditions may have the potential to select for *L. monocytogenes* with increased acid tolerance, increased tolerance towards other stresses and increased virulence (21). However, the nisin-resistant mutant of *L. monocytogenes* Scott A isolated at pH 5.6 failed to display constitutively expressed acid tolerance. Moreover, this finding excludes a possible involvement of acid tolerance in the nisin resistance mechanism of this strain by retarding dissipation of the PMF. It could be envisaged that constitutively expressed acid tolerance coincides with increased capability of maintaining a high intracellular pH due to increased ATPase activity, the main regulator of the intracellular pH in *L. monocytogenes* Scott A (26).

In conclusion, nisin resistance in *L. monocytogenes* may be based on (a combination of) several mechanisms which relate back to alterations in the cell wall, changes in the cytoplasmic membrane or other so far not-identified mechanisms. No correlation exists between nisin

susceptibility and pediocin susceptibility for the nine strains described in the present study and, moreover, nisin and pediocin are more antilisterial in combination than individually. Therefore, considerable promise remains for the use of nisin and pediocin in a cocktail for extending the shelf life and increasing the safety of ready-to-eat foods. In combination with other common food preservation techniques (low pH, low temperature, high salt) the development of nisin resistance might be further reduced.

Acknowledgements

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10

Summary and concluding remarks

Introduction

Prevailing consumer requirements for minimally processed convenience foods without chemical preservatives coupled with an extended shelf-life has raised the introduction of ready-to-eat foods which rely on refrigeration as the main preservation technique. Those foods leave only a small margin of safety with respect to pathogenic and spoilage microorganisms. *L. monocytogenes* is of particular concern in those food products due to its wide distribution in the environment and consequently its presence on raw and minimally processed foods, and its ability to grow at refrigeration temperature. Other worries for the food industry with this pathogen are its growth at high osmotic strength and its ability to form biofilms on food-processing equipment (12, 18). Although other pathogens may often be more prevalent on certain foods, the consequences of a single *L. monocytogenes* epidemic both in terms of mortality and economic impact are serious enough to render this pathogen as an important threat to consumers and the food industry. Understanding of the growth and survival of *L. monocytogenes* and knowledge of the interaction between *L. monocytogenes* and other microorganisms will contribute to the design of adequate safety measures. This thesis deals with the physiology of *L. monocytogenes*, with reference to conditions to be encountered in foods. Three major aspects are covered, *i.e.*, peptide utilization, osmoregulation and nisin resistance.

Role of peptides during growth of *L. monocytogenes*

Outbreaks of listeriosis have mainly been traced back to the consumption of raw vegetables, meat and milk products, that contain very low amounts of free amino acids. Considering that *L. monocytogenes* is auxotroph for the amino acids leucine, isoleucine, valine, methionine and cysteine (30), the pathogen needs alternative nitrogen sources for growth to high cell densities in those food products. *L. monocytogenes* has the ability to produce an extracellular metalloprotease (Mpl), which is however only produced at 37°C and moreover in very low amounts (4). Mpl is one of the virulence factors of *L. monocytogenes* and is involved in the processing of phosphatidylcholine-specific phospholipase from the precursor to its active form. This phospholipase is consecutively involved in the disruption of the double membrane which is formed around the bacterium when it enters a neighbouring mammalian cell (9, 20, 23, 29, 31). The supply of essential amino acids for growth of *L. monocytogenes* in most foods therefore has to originate from other sources.

In this thesis it is demonstrated that *L. monocytogenes* benefits from the proteolytic activity of other microorganisms or enzymes in a medium with the milk protein casein present as the sole source of nitrogen (Chapter 3). As a consequence of proteolytic breakdown of proteins, a mixture of peptides of different lengths and some amino acids will become available, which makes it particularly worthwhile to examine the nutritional value of peptides. For effective utilization of peptides, *L. monocytogenes* is equipped with two different peptide transport systems. The first one is a proton motive force (PMF) driven transport system that allows transport of various di- and tripeptides (Chapter 2). The second one is an ATP-dependent oligopeptide uptake system, which is capable of transporting

peptides up to at least eight residues (Chapter 3). Peptidase activities were detected in cell extracts of *L. monocytogenes* and were absent in the supernatant, indicating that following internalization, the peptides are degraded by peptidases to supply *L. monocytogenes* with amino acids for growth. The peptide transporters of non-proteolytic *L. monocytogenes* play a crucial role in the amino acid supply of the pathogen, which is schematically presented in Figure 1.

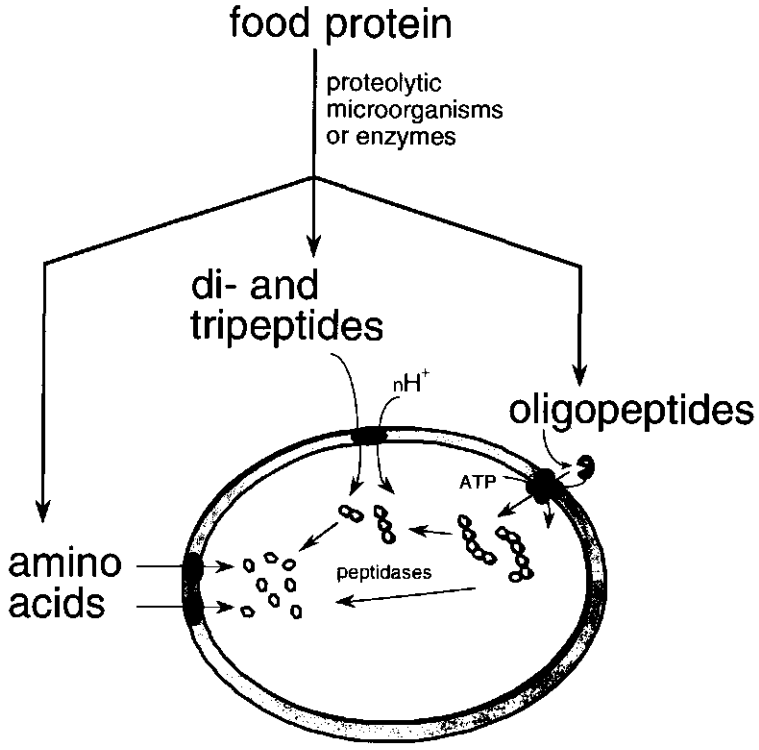

Figure 1. Model for the proteolytic system of *L. monocytogenes*. The lack of an active protease and its inability to synthesize all the amino acids from inorganic ammonium salts, make *L. monocytogenes* dependent on indigenous proteinases and the proteolytic activity of other microorganism present in foods. The hydrolyzed products may enter via amino acid transporters (16, 34), a PMF-driven di-tripeptide carrier (36) or via an ATP-dependent oligopeptide uptake system (37). Accumulated peptides are degraded by intracellularly located peptidases to amino acids necessary for protein synthesis.

A study of Marquis *et al.* (20) has demonstrated that amino acid deprivation during intracellular growth of *L. monocytogenes* was alleviated by the addition of peptides, which suggests that the peptide transport systems of *L. monocytogenes* may in addition have a role during intracytoplasmic growth of the pathogen in mammalian cells. During intracellular

growth, the acquisition of nutrients is an important factor in the bacterial capacity to compete with the host cell. Recently, Coffey *et al.* (4) isolated and characterized the metalloprotease of *L. monocytogenes*. It was demonstrated that the protease degraded ß-casein as well as actin. The latter protein is relatively abundant in the immediate vicinity of *L. monocytogenes* during intracellular growth and within the double membraned phagosome during cell-to-cell spread. Thus, although Mpl has no significance in foods, it may be of considerable importance in the formation of peptides and amino acids for intracellular growth of *L. monocytogenes*.

In addition to their role in nitrogen metabolism, amino acids and peptides are accumulated by *L. monocytogenes* as a mechanism of maintaining turgor (Chapter 4). The growth of osmotically stressed *L. monocytogenes* is strongly stimulated by the addition of peptone. Cells grown with peptone accumulated substantial pools of amino acids and peptides from the medium such that these pools contribute to osmoregulation. Particularly, the individual peptides prolyl-hydroxyproline, prolyl-glycine and prolyl-glycyl-glycine were very effective in conferring osmotolerance and could substitute for the conventional compatible solute betaine. Given the prevalence of these peptides in collagen, the growth of *L. monocytogenes* might be stimulated by the accumulation of these peptides in especially low a_w meat products, like salami and other fermented sausages. This is the first description of accumulation of peptides from the growth medium during osmoregulation. Although the accumulation of peptides as contributors to the restoration of turgor pressure has been noted before in a variety of species, these peptides were derived by synthesis from the free amino acids in the cytoplasm (10, 22, 33). In addition, these results indicate that the di-tripeptide carrier, which displays a high affinity for various proline-containing peptides (Chapter 2), may not only have a function in supplementation of nutrients for protein synthesis but also in osmoregulation of *L. monocytogenes*.

Betaine and L-carnitine transport

Betaine and carnitine can contribute significantly to growth of *L. monocytogenes* at high osmolarity (2, 28). The osmoprotective capacity of betaine is well-known among prokaryotic organisms, wheras L-carnitine is only recognized as a compatible solute in the gram-positive bacteria *L. monocytogenes* (2), *L. plantarum* (15) and the gram-negative *Escherichia coli* (Chapter 7, see below). Betaine occurs in high concentrations in foods originating from plants whereas foods of animal origin generally have a high carnitine content. In Chapter 5 the transport of L-carnitine in *L. monocytogenes* is described in detail. Uptake of L-carnitine is mediated by a constitutively expressed transporter that is energized by ATP hydrolysis. Analysis of cell extracts revealed that L-carnitine was not further metabolized, which supplies further evidence for its role as an osmoprotectant in *L. monocytogenes*. L-carnitine is taken up via a high-affinity tranporter with a K_m^{app} of 10 μM and a maximum rate of transport (V_{max}) of 48 nmol min^{-1} mg of $protein^{-1}$. Competition experiments revealed that the L-carnitine transporter has high affinity for L-carnitine, acetylcarnitine, and γ-butyrobetaine, and shows neglible affinity for L-proline and betaine. The latter result initiated a study on

the regulation of betaine and L-carnitine transport in *L. monocytogenes*, which is described in Chapter 6. The data demonstrate that betaine enters *L. monocytogenes* cells via a secondary transport system (K_m^{app}, 10 μM; V_{max}, 56 nmol min^{-1} mg of $protein^{-1}$) and noteworthy, betaine and L-carnitine enter the cytoplasm solely via distinct transporters in the pathogen. Recently, Gerhardt *et al.* (13) reported that *L. monocytogenes* DP-L1044 possesses a specific betaine transporter which is sodium dependent (K_m for Na^+, 200 mM). However, other observations from the same laboratory showed that betaine transport in *L. monocytogenes* Scott A proceeds without sodium (17). Likewise, our results give no indications for cotransport of betaine with such a poor affinity for Na^+ in *L. monocytogenes* Scott A, since we demonstrated that in the presence of sodium neither stimulation nor inhibition of betaine uptake occurred. Moreover, the results where comparable irrespective whether osmotic stress was imposed by KCl rather than NaCl.

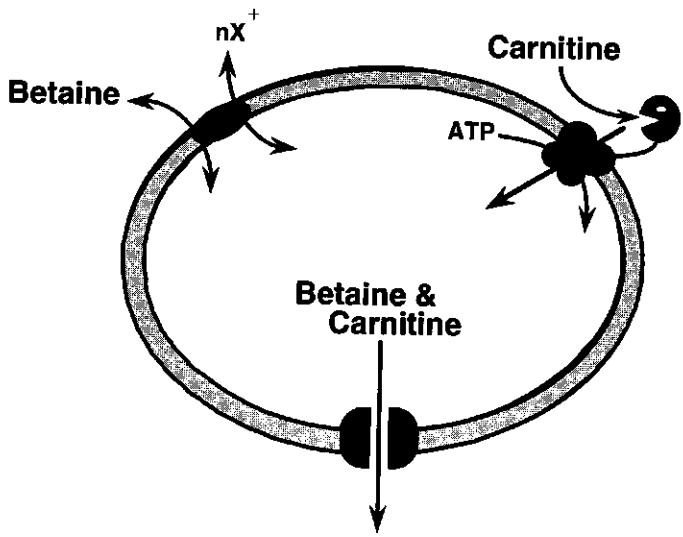

Figure 2. Uptake and efflux systems for betaine and L-carnitine in *L. monocytogenes*. Betaine and L-carnitine enter the cytoplasm of *L. monocytogenes* via distinct transport systems. Betaine is taken up via a reversible secondary transporter and L-carnitine transport proceeds via an ATP-dependent uptake system. X = H^+ or Na^+, see text for details. Upon an osmotic downshock, betaine and L-carnitine are rapidly released to the external medium through activation of protein channels in the cytoplasmic membrane.

The initial uptake rates of betaine and L-carnitine are not influenced by an osmotic upshock, but the duration of transport of both osmolytes is directly related to the osmotic strength of the medium. Regulation of both betaine and L-carnitine uptake is subject to *trans* inhibition by preaccumulated solute. Importantly, internal betaine not only inhibits the

transport of external betaine but also that of L-carnitine and, similarly, internal L-carnitine inhibits both betaine and L-carnitine transport. The *trans* inhibition is alleviated upon osmotic upshock which suggests that alterations in membrane structure are transmitted to the binding pockets for betaine and L-carnitine of both transporters at the inner surface of the membrane. The osmotic regulation of betaine and L-carnitine uptake represents a novel osmolyte sensing mechanism. Upon osmotic downshock, betaine and L-carnitine are rapidly released by *L. monocytogenes* as a consequence of activation of a channel-like activity. The transport mechanisms involved in the uptake and efflux of betaine and L-carnitine in *L. monocytogenes* are depicted in Figure 2.

The findings also implicate that the transport activities of the betaine and L-carnitine transporters of *L. monocytogenes* are rather high under conditions of low osmolarity, which is an interesting observation that has so far not been seen for any other osmolyte transport system in bacteria. The accumulation of betaine and L-carnitine might possibly be a general response to conditions of stress (high osmolarity, low pH, anaerobiosis, low temperature). Indeed, the intracellular pools of these compounds in cells grown under anaerobic conditions were higher than those under aerobic conditions and betaine and L-carnitine are accumulated to much higher concentrations in cells grown at 7°C compared to cells grown at 30°C (35). Moreover, *L. monocytogenes* cells grown at 7°C take up L-carnitine with a relatively high rate (Chapter 5), particularly when compared with the uptake rate of for example the amino acid L-lysine (Fig. 3). Data of Ko *et al.* (17) and Smith (32) have demonstrated that betaine and L-carnitine stimulate the growth of *L. monocytogenes* at low temperature, confirming the role of betaine and L-carnitine in cold tolerance of the psychrotrophic *L. monocytogenes*.

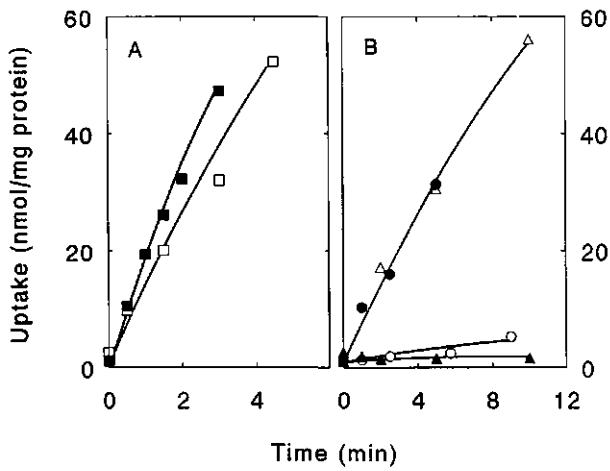

Figure 3. Uptake of L-carnitine and L-lysine in *L. monocytogenes*. Cells were grown at 30°C (panel A) or at 7°C (panel B) and transport assays were made at 30°C and 5°C. (■,●), transport of L-carnitine at 30°C; (□,○), transport of L-lysine at 30°C; (Δ), transport of L-carnitine at 5°C, and (▲), transport of L-lysine at 5°.

Considering the crucial role of L-carnitine in the ß-oxidation of fatty acids in the mitochondria, mammalian cells will contain high levels of carnitine and acetylcarnitine. The L-carnitine transporter may therefore facilitate the intracellular growth of *L. monocytogenes* and thus play a role in the virulence of the pathogen.

Chapter 7 is devoted to the role of L-carnitine in the osmotic stress response of the gram negative *E. coli* with linkage to enterohaemorrhagic *E. coli* O157:H7. Carnitine enhanced the growth of *E. coli* O157:H7 in a medium of otherwise inhibitory osmotic strength. Its osmoprotective ability is comparable with that of betaine. Since carnitine is an important compound in mammalian tissues it might play a role in the growth of the pathogen on low a_w meat products. Using specific uptake mutants of *E. coli* K-12, it was established that under osmotic stress carnitine accumulates in the cytoplasm following import through the ProP and ProU transport systems. Betaine and carnitine also protect *E. coli* cells while growing anaerobically at inhibitory osmolarity, which seems of considerable importance in vacuum and modified packaged foods and in view of the occurrence of *E. coli* in the intestine where anaerobic conditions prevail. Under anaerobic conditions, an *E. coli* K-12 strain with lesions in both *proP* and *proU*, accumulates low levels of L-carnitine but fails to accumulate betaine when these compounds are supplied in the external medium. This is probably a result of uptake of L-carnitine by the secundairy transporter CaiT. The *caiT* gene forms part of the *caiTABCDE* operon which encodes the carnitine pathway (11), and is transcribed during anaerobic growth in the presence of carnitine. However, further experiments revealed that the carnitine pathway, including CaiT, does not play a significant role in osmoregulation of *E. coli* during anaerobiosis. Together, the results indicate that ProP and ProU are the sole transport systems involved in carnitine influx both in aerobically and anaerobically osmotically stressed *E. coli* cells.

Nisin resistance mechanisms in *L. monocytogenes*

The application of bacteriocins like nisin as an extra safety hurdle in ready-to-eat foods might introduce the emergence of nisin-resistant mutants of *L. monocytogenes*. Understanding of how bacteria become resistant to bacteriocins may prevent their misapplication and guarantees awareness of conditions which promote selection of pathogens with high intrinsic resistance.

Chapter 8 supplies evidence for a reduction in the diphosphatidylglycerol (DPG) content of the cytoplasmic membrane of a nisin-resistant variant (NIS^R) of *L. monocytogenes* Scott A which was isolated by stepwise exposure to increasing concentrations of nisin in brain heart infusion (BHI) broth. The NIS^R strain was about 12 times more resistant to nisin than the wild-type (WT) strain. Accordingly, higher nisin concentrations were required to dissipate both components of the proton motive force in the NIS^R strain as compared to the WT strain. Comparison of the membrane fatty acyl composition of the sensitive strain with that of its NIS^R derivative revealed no significant differences. From phospholipid headgroup composition analysis and phospholipid biosynthesis measurements during growth in the absence and presence of nisin, it could be inferred that the NIS^R strain produces relatively

more phosphatidylglycerol (PG) and less DPG in comparison with the parent strain. Monolayer studies using pure lipid extracts from both strains showed that nisin interacted more efficiently with lipids derived from the WT strain than those of the NIS^R strain, reflecting qualitative differences in nisin sensitivity. Involvement of the cell wall in acquisition of nisin resistance is unlikely, since WT and NIS^R showed a comparable sensitivity to lysozyme. Recently, it has been demonstrated that nisin penetrates more deeply into lipid monolayers of DPG than those of other lipids including PG, phosphatidylcholine (PC), phosphatidylethanolamine (PE), monogalactosyldiacylglycerol and digalactosyldiacylglycerol (8), which rationalizes our findings.

Following this study, a number of phenotypic characters of five nisin-resistant *L. monocytogenes* strains, including the NIS^R of *L. monocytogenes* Scott A, were compared with their parental strains (Chapter 9) in order to provide a better base of knowledge for the application of nisin in foods. The strains gained nisin resistance through serial passages in nisin-containing medium. Two of the five nisin-resistant variants originate from the same parent strain (Scott A), one is the abovementioned NIS^R strain which was isolated at pH 7.2 and the other was isolated at pH 5.6. The other nisin-resistant variants were isolated at pH 7.2 and are derivatives of *L. monocytogenes* strains 4B, 669, and 13, which are isolates from food or food environments. At pH 7.2, the nisin-resistant variant of strain 669 as well as the nisin-resistant mutant of Scott A that was isolated at pH 5.6 grew slower than their nisin-sensitive counterparts both at 7°C and 30°C. The other three nisin-resistant strains exhibited similar growth rates as their parent strains at both temperatures. The resistant variants of strain 13 and 669 grew much slower at pH 5.6 compared to the original strains. The nisin-resistant variants of strain 4B, 13 and 669 showed a reduced sensitivity towards the peptidoglycan hydrolytic enzyme mutanolysin compared with their nisin-sensitive wild-type strains, whereas the nisin-resistant variant of *L. monocytogenes* Scott A which was trained to nisin at pH 5.6 showed an increased sensitivity to mutanolysin in comparison with its parent strain. The *L. monocytogenes* strains manifested completely unpredictable susceptibility towards pediocin. The nisin-sensitive strains were either sensitive or resistant to pediocin and the same was valid for the nisin-resistant strains without indications for either cross-sensitivity or cross-resistance to nisin and pediocin. The results show that the mechanism by which *L. monocytogenes* gaines nisin resistance may vary among strains and may also be dependent on the conditions under which the strain acquired the resistance. It was concluded that nisin resistance in *L. monocytogenes* is a complex multifaceted phenomenon which may be based on (a combination of) several mechanisms. It can be imagined that nisin resistance can originate from alterations in the cell wall, the cell membrane and the cytoplasm (Table 1). Alterations in the cell wall may affect the presence or accessability of attachment sites hindering the incorporation of nisin into the membrane to produce pores. Nisin-resistant variants of *L. innocua* isolated by Maisnier-Patin and Richard (19) showed increased cell wall hydrophobicity, resistance to phage attack and three cell-wall acting enzymes as well as peptidoglycan hydrolytic enzymes lysozyme and mutanolysin as compared to the parental strains. The changes were attributed to abnormal cell wall synthesis and autolysin inhibition. For a nisin-resistant mutant of *L. monocytogenes* F6861 it is reported that the acquired

resistance originates from reduced cell wall hydrophobicity (5, 6). Breuer and Radler (3) provided evidence that nisin resistance in a *Lactobacillus casei* strain was associated with modifications of cell wall polysaccharides. The resistant variant produced larger amounts of phosphate containing anionic polysaccharides composed of rhamnose and galactose residues. Alterations in the phospholipid content of the cytoplasmic membrane can also affect the association and/or penetration of nisin in the cytoplasmic membrane of *L. monocytogenes* membranes thereby modulating nisin sensitivity (25, 38). Alternatively, changes in the fatty acid composition of *L. monocytogenes* strains have been reported (21, 24). Modifications in the fatty acid composition of the cytoplasmic membrane apparently result in a more rigid membrane thereby hindering nisin insertion. The overall membrane constitution can possibly also play a role in nisin sensitivity as became evident from a study in which two *Lactobacillus* and *Pediococcus* strains that manifest over hundred-fold differences in nisin sensitivity were compared (1). Finally, it is conceivable that changes in the cytoplasm confer resistance to nisin as a result from the induction of proteins that indirectly fight the harmful effects of nisin. Membrane permeabilization by nisin will results in dissipation of the ph gradient (or PMF) resulting in exposure of the cytoplasm to a low pH. Resistance to nisin could therefore be acquired through induction of chaperons which would prevent the denaturation of vital enzymes by low pH as has been demonstrated for other environmental stresses (14). Moreover, *de novo* protein synthesis has recently been demonstrated to play a key role in the development of pH homeostasis during the acid tolerance response of *L. monocytogenes* (27). It can therefore be imagined that similar processes are involved in the acquisition of nisin resistance resulting in the production of chaperons and increased capability of maintaining intracellular pH.

Table 1. Possibilities for nisin resistance mechanisms in *L. monocytogenes*

Level	Change(s)	Reference
1. cell wall	thickening cell wall (abnormal cell wall synthesis; autolysin inhibition)	19
	reduced hydrophobicity	5, 6
	modifications cell wall polysaccharides	3
2. cell membrane	fatty acid composition	21, 24
	phospholipid composition	25, 38
	overall membrane constitution	1
3. cytoplasm	capability of maintaining intracellular pH	
	induction of stress proteins	

Chapter 9 also reveals that all resistant strains became increasingly sensitive to nisin at pH 5.6, a representative pH for most foods, as compared to pH 7.2. In addition, with skimmed milk as a model, it was shown that nisin and pediocin are more effective in a mixture than individually against nisin-sensitive and nisin-resistant *L. monocytogenes* Scott A. De Martinis *et al.* (7) recently demonstrated that a combination of traditional food-preservation hurdles (low pH, low temperature, high salt) diminishes the likelihood of nisin resistant variants to emerge, whereas those factors on their own did not reduce the frequency at which resistance arises. Taking the above into account and considering that low pH conditions have the potential to select for *L. monocytogenes* with increased acid tolerance and increased tolerance towards other stresses (26), it is advisable to use nisin in a cocktail with other bacteriocins (*e.g.* pediocin) or in combination with other mild preservation techniques like low pH in order to prevent the selection for multiresistant listeriae.

In conclusion, the research described in this thesis has significantly improved the understanding of the physiology of the important foodborne pathogen *L. monocytogenes*. The results implicate that specific components of the food matrix have an important bearing on survival and growth of *L. monocytogenes* and present new insights into factors that contribute to its success as a foodborne pathogen. This knowledge is useful in the development of new preservation procedures and products for which the pathogen is of concern.

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Samenvatting

De toenemende vraag van consumenten naar gemaksvoeding zonder chemische conserveringsmiddelen, heeft geleid tot de introductie van diverse kant-en-klaar maaltijden die na productie voornamelijk geconserveerd worden via koeling. Deze producten bevatten rauwe of minimaal verhitte bestanddelen, hetgeen de aanwezigheid van diverse micro-organismen impliceert. Een bacterie die algemeen in de natuur voorkomt, is de voedselpathogeen *Listeria monocytogenes*. Deze pathogeen kan listeriose veroorzaken, een ziekte die onder andere kan leiden tot hersenvliesontsteking met een dodelijke afloop in circa 25% van de gevallen. De meeste infecties treden op bij personen met een verzwakt afweersysteem. De consequentie van listeriose bij zwangere vrouwen is vaak een spontane abortus. *L. monocytogenes* kan groeien in de koelkast en vormt daardoor een potentieel gevaar voor bovengenoemde kant-en-klaar maaltijden. Andere factoren die bijdragen aan de persistentie van de bacterie in de voedselketen zijn het vermogen om te groeien bij relatief hoge zoutconcentraties en om biofilms te vormen op diverse oppervlakken. Kennis omtrent de groei en overleving van *L. monocytogenes* in voedselmatrices en inzicht in de interactie tussen de pathogeen en andere bacteriën zijn essentieel teneinde adequate veiligheidsmaatregelen te kunnen nemen. Deze conserveringsmethoden dienen mild te zijn teneinde te voldoen aan de eisen die de consument aan levensmiddelen stelt (natuurlijk en vers). Voorbeelden van milde bioconserveringstechnieken zijn het verpakken onder gewijzigde gascondities of het toevoegen van bacteriocines die geproduceerd worden door melkzuurbacteriën. Dit proefschrift beschrijft achtereenvolgens de rol van peptiden in de groei van *L. monocytogenes*, osmoregulatie door betaine en carnitine in *L. monocytogenes* en tenslotte wordt aandacht besteed aan het optreden van bacteriocineresistentie in *L. monocytogenes*.

Hoofstuk 2 en 3 geven een gedetailleerde beschrijving van het vermogen van *L. monocytogenes* om peptiden te gebruiken als bron van essentiële aminozuren. *L. monocytogenes* kan geen eiwitten afbreken en heeft bovendien een vijftal aminozuren nodig om te groeien. Aangetoond werd dat de pathogeen twee verschillende systemen heeft om peptiden de cel in te transporteren. Di- and tripeptiden worden opgenomen via een systeem dat gedreven wordt door de electrochemische gradient van protonen over de cytoplasmatische membraan (de protonen drijvende kracht). Het tweede systeem, dat oligopeptiden met een ketenlengte tot acht aminozuurresiduen kan transporteren, heeft ATP nodig om te functioneren. In het cytoplasma bevinden zich verschillende peptidases, waarmee *L. monocytogenes* de peptiden na opname splitst in aminozuren om deze vervolgens te gebruiken voor groei. De peptide transportsystemen stellen de pathogeen in staat om te groeien in levensmiddelen die weinig vrije aminozuren bevatten maar wel peptiden ten gevolge van inwerking van proteases (endogeen of afkomstig van andere micro-organismen in het levensmiddel). Aangetoond werd dat bacteriën die samen met *L. monocytogenes* kunnen voorkomen in bijvoorbeeld melk (*Bacillus cereus* en *Pseudomonas fragi*) de groei van *L. monocytogenes* inderdaad kunnen stimuleren. De peptide transportsystemen van *L. monocytogenes* kunnen mogelijk tevens een bijdrage leveren aan de intracellulaire groei van de pathogeen in dierlijke cellen.

Hoofdstuk 4 beschrijft de rol van aminozuren en peptiden in de groei van *L.*

monocytogenes bij een hoge zoutconcentratie. De groei van de pathogeen in een minimaal medium met 0.625 M NaCl, wordt sterk gestimuleerd door toevoeging van pepton. Onder deze condities worden substantiële hoeveelheden aminozuren en peptiden aangetroffen in het cytoplasma, die een bijdrage leveren aan de osmoregulatie van *L. monocytogenes*. Met name glycine- en prolinebevattende peptiden blijken de groei van de pathogeen onder osmotische stress te stimuleren. Aangezien collageen veel prolyl-glycine en hydroxyproline-glycine bindingen bevat, kan de accumulatie van dergelijke peptiden in *L. monocytogenes* een belangrijke betekenis hebben voor de groei van de pathogeen in bepaalde vleesproducten.

Betaine en carnitine zijn componenten waarvan recentelijk werd aangetoond dat ze een belangrijke bijdrage kunnen leveren aan de groei van *L. monocytogenes* in een medium met een hoge osmolariteit. Betaine en carnitine zijn met name aanwezig in levensmiddelen van respectievelijk plantaardige en dierlijke oorsprong. De beschermende werking van betaine onder osmotische stress is alom bekend bij prokaryoten, terwijl een dergelijke werking van carnitine alleen bekend is voor *L. monocytogenes, Lactobacillus plantarum* en *Escherichia coli* (beschreven in Hoofdstuk 7, zie volgende paragraaf). Hoofdstuk 5 geeft een gedetailleerde karakterisatie van L-carnitine transport in *L. monocytogenes*. Het transportsysteem voor L-carnitine is constitutief aanwezig en wordt geënergeerd via ATP hydrolyse. Het systeem heeft een hoge affiniteit voor L-carnitine (K_m^{app} is 10 μM) en de maximale transportsnelheid (V_{max}) bedraagt 48 nmol per min per mg eiwit. Competitie experimenten hebben aangetoond dat het transportsysteem affiniteit vertoont voor L-carnitine, acetylcarnitine en τ-butyrobetaine, maar geen affiniteit heeft voor proline en betaine. Dit laatste gegeven initieerde een studie naar de regulatie van betaine en L-carnitine transport, de resultaten hiervan zijn beschreven in Hoofdstuk 6. Aangetoond werd dat het transport van betaine en L-carnitine in *L. monocytogenes* verloopt via twee afzonderlijke systemen. De initiële opnamesnelheden van betaine en L-carnitine worden niet beïnvloed wanneer de osmolariteit van het medium waarin de cellen zich bevinden ineens sterk wordt verhoogd. De duur van het transport van beide stoffen is echter direct gerelateerd aan de osmotische sterkte van het medium. Bovendien is gevonden dat de opname van zowel betaine als L-carnitine onderhevig is aan *trans* inhibitie. De aanwezigheid van betaine in de cel remt niet alleen het transport van betaine de cel in, maar remt tevens het transport van L-carnitine de cel in. Evenzo remt L-carnitine aan de cytoplasmatische kant van het membraan, het transport van zowel L-carnitine als betaine. De *trans* inhibitie wordt opgeheven bij een plotseling toename in de osmolariteit van het medium hetgeen waarschijnlijk het gevolg is van veranderingen in membraanstructuur waardoor de conformatie van de transportereiwitten wordt beïnvloed. Dit vertegenwoordigt een nieuw osmotisch regulatiemechanisme dat niet eerder is aangetoond bij andere bacteriën. Bij een plotselinge afname in de osmotische druk, worden zowel betaine als L-carnitine met hoge snelheid uitgescheiden, waarschijnlijk via activatie van eiwitkanalen in het cytoplasmatische membraan.

Hoofdstuk 7 is gewijd aan de rol van carnitine tijdens de groei van *E. coli* onder osmotische stress in relatie tot de voedselpathogeen *E. coli* O157:H7. Aangetoond werd dat carnitine de groei van *E. coli* O157:H7 stimuleert in een medium met een hoge osmolariteit. De osmoprotectieve werking van carnitine was vergelijkbaar met die van betaine. Omdat

carnitine een belangrijke component is in dierlijk weefsel, zou het kunnen bijdragen aan de groei van *E. coli* O157:H7 in (gefermenteerde) vleesproducten. Gebruikmakend van opnamemutanten van *E. coli* K-12 werd aangetoond dat de accumulatie van carnitine optreedt via de ProP and ProU systemen, die tevens verantwoordelijk zijn voor de import van betaine and proline. Onder anaërobe condities, accumuleert een *E. coli* mutant waarbij ProP en ProU zijn uitgeschakeld, lage concentraties L-carnitine in het cytoplasma. Dit is waarschijnlijk het resultaat van opname van carnitine via het transportsysteem CaiT; het *caiT* gen maakt deel uit van het *caiTABCDE* operon dat tot expressie komt tijdens anaërobe groei in aanwezigheid van carnitine. Vervolgexperimenten hebben echter aangetoond dat CaiT geen belangrijke rol speelt in de osmoregulatie van *E. coli* tijdens anaërobe groei.

De introductie van bacteriocines zoals nisine als een extra veiligheidsmaatregel in kant-en-klaar maaltijden zou samen kunnen gaan met het ontstaan van nisineresistente mutanten van *L. monocytogenes*. Begrip van bacteriocine resistentiemechanismen kan voorzien in een rationele basis voor het gebruik van bacteriocines. Hoofdstuk 8 beschrijft het mechanisme van nisineresistentie in een stam van *L. monocytogenes* (Scott A). Deze stam werd verkregen via blootstelling aan toenemende concentraties nisine; de nisine concentratie die nodig is om deze stam te remmen in de groei was ongeveer 12 keer zo hoog als de nisine concentratie die nodig is om de oorspronkelijke stam te remmen. Aangetoond werd dat in het geval van de resistente mutant, hogere nisine concentraties nodig waren om de dissipatie van zowel de membraanpotentiaal als de transmembranaire pH-gradient te bewerkstelligen. De vetzuursamenstelling van de membraan van de gevoelige stam verschilde niet significant met die van de nisineresistente mutant. Via vergelijking van de phospholipide kopgroepsamenstelling en bestudering van de phospholipide biosynthese tijdens groei in de aan- en afwezigheid van nisine, kon worden afgeleid dat de nisineresistente stam relatief meer phosphatidylglycerol (PG) en minder diphosphatidylglycerol (DPG) produceert in vergelijking met de originele stam. Monolaagstudies toonden vervolgens aan dat nisine een sterkere interactie aangaat met lipiden van de gevoelige stam dan met die van de resistente stam, hetgeen kwalitatief de gevoeligheid voor nisine reflecteert. Volgend op deze studie werden in Hoofdstuk 9 een aantal fenotypische karakteristieken van vijf nisineresistente stammen bestudeerd. Alle stammen werden geïsoleerd via stapsgewijze blootststelling aan nisine. Bij pH 7.2, groeiden twee van de nisineresistente varianten langzamer dan hun nisinegevoelige tegenhangers zowel bij 7°C als bij 30°C, terwijl de andere drie nisineresistente stammen dezelfde groeikarakteristieken vertoonden als de orginele stammen. Eén van deze drie stammen groeide echter veel langzamer bij pH 5.6 dan de oorspronkelijke nisinegevoelige stam. Drie van de vijf nisineresistente varianten vertoonden een verminderde gevoeligheid voor het celwandafbrekende enzym mutanolysine in vergelijking met de corresponderende nisinegevoelige stammen, terwijl één van de nisin-resistente stammen juist gevoeliger was voor mutanolysine dan de oorspronkelijke stam. De gevoeligheid van de *L. monocytogenes* stammen voor pediocine bleek onvoorspelbaar; de nisinegevoelige stammen waren gevoelig of niet gevoelig voor pediocine en hetzelfde gold voor de nisineresistente stammen. Deze resultaten laten zien dat het mechanisme van nisineresistentie waarschijnlijk stam-afhankelijk is en suggereert dat nisineresistentie in *L. monocytogenes* complex is. Alle nisineresistente

stammen vertonen bij een pH van 5.6, hetgeen een representatieve pH voor veel levensmiddelen is, een verhoogde gevoeligheid voor nisine. Met magere melk als een modelsysteem werd aangetoond dat nisine en pediocine in een mengsel effectiever zijn dan afzonderlijk tegen de nisinegevoelige en nisineresistente *L. monocytogenes* Scott A. Het toepassen van nisine in een cocktail met andere bacteriocines of in combinatie met andere milde conserveringstechnieken zoals een lage pH kan daarom wellicht een efficiënte conserveringsstrategie inhouden.

Het onderzoek dat beschreven is in dit proefschrift heeft een belangrijke bijdrage geleverd aan het begrip van de fysiologie van de voedselpathogeen *L. monocytogenes*. De resultaten tonen aan dat bepaalde componenten van de voedselmatrix kunnen bijdragen aan de groei en overleving van *L. monocytogenes* en hebben diverse nieuwe inzichten aan het licht gebracht. De verkregen kennis is ondermeer bruikbaar bij het ontwikkelen van nieuwe conserveringstechnieken en producten waarin deze pathogeen van belang is.

Curriculum Vitae

Annette Verheul werd geboren op 29 juni 1966 te Giessenburg. In juni 1984 werd het VWO diploma behaald aan de Christelijke Scholengemeenschap "Oude Hoven" te Gorinchem. Vervolgens studeerde zij 2 jaar Scheikunde aan de Rijksuniversiteit te Utrecht en 5 jaar Levensmiddelentechnologie aan de Landbouwuniversiteit te Wageningen. In augustus 1991 werd het doctoraalexamen Levensmiddelentechnologie behaald met als afstudeervakken Levensmiddelenchemie en Proceskunde. Stages werden doorlopen aan de University of Nottingham (Department of Applied Biochemistry and Food Science) in Engeland en op de afdeling Fermentation and Downstream Research van Quest International Nederland B. V. te Naarden. Van december 1991 tot maart 1993 werkte zij als projectmedewerker Bioprocestechnologie aan de Hogeschool Heerlen aan het opzetten van de European Postgraduate Diploma Course Biotechnology. In maart 1993 trad zij in dienst als Assistent in Opleiding bij de vakgroep Levensmiddelentechnologie, sectie Levensmiddelenchemie en -microbiologie, waar het in dit proefschrift beschreven onderzoek werd verricht. Het onderzoek vond plaats in het kader van een EU project getiteld "Improvement of the safety and quality of refrigerated ready to eat foods using novel mild preservation techniques", waarin 2 universiteiten, 5 onderzoeksinstituten en 2 bedrijven participeerden uit 5 EU lidstaten. Sinds juni 1997 is zij werkzaam als wetenschappelijk medewerker binnen de afdeling Microbiologie, sectie Aroma en Starters, van het Nederlands Instituut voor Zuivelonderzoek te Ede.

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